Supporting Information

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SI Materials and Methods

Taxonomic Coverage, Nucleic Acid Isolation, and Sequencing. In addition to bats, we also sequenced the cat (*Felis catus*), rabbit (*Oryctolagus cuniculus*), and pig (*Sus scrofa*). Accession numbers for all new sequences are EU914923–EU914937. For additional outgroups we also obtained the published sequences for human (*Homo sapiens*; NM_198999), mouse (*Mus musculus*; NM_030727), rat (*Rattus norvegicus*; NM_030840), gerbil (*Meriones unguiculatus*; AF230376), cow (*Bos taurus*; XM_616468), and dog (*Canis familiaris*; XM_540393), and we used BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat) searching to identify *Prestin* from the horse (*Equus caballus*) genome.

To obtain the coding sequence of the *Prestin* gene from *Rhinolophus ferrumequinum*, *Rousettus leschenaulti*, and *Myotis ricketti*, we carried out 5′- and 3′-rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA Amplification kit (Clontech). Gene-specific primers for 5′-RACE (GSP1: 5′-CAT TAA ACT CCT TGC CAC CCA ACA GC-3′ and NGSP1: 5′-CTG AAC AAG GCT TCG AGA CAA GGA G-3′) and 3′-RACE (GSP2: 5′-GGT GTC TGT AGG TTT GGA TTT GTG GC-3′ and NGSP2: 5′-ACC TCA ACG TGT GTT CCC TAG GCG-3′) were designed from conserved *Prestin* sequences in human, mouse, rat, dog, and cow. Note, the beginning of our coding sequences in bats was found to correspond to exon 3 in the human. Therefore we use the numbering of exons as they apply to the human sequence, and, on the basis of coding regions alone, we cannot rule out the possibility that the exact number of exons in bats differs from that of humans. From the whole cDNA sequences, we designed the primers Fu (5′-CAG AGG RCY ATG GAT CAT GCT GAA G-3′) and Ru (5′-TCA TTC ACC CTC CAA ATC AAG C-3′) and undertook RT-PCR to amplify the *Prestin* gene in the other bat species and cat, pig, and rabbit. For first-strand cDNA synthesis, 2.5 μg of total RNA was reverse transcribed with SuperScript III (Invitrogen) in a volume of 20 μl and stored at −20°C for further use. Overlapping products from exon 3 to exon 11 and exon 8 to exon 20 were amplified with the primer pairs of Fu-GSP1 and NGSP2-Ru, respectively. 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 using Big Dye Terminator kits (Applied Biosystems) on an ABI 3730 automated DNA sequencer. To avoid artifacts, multiple clones (7–12) were sequenced for every specimen.

Splice Variants. To test for the existence of splice variants specific to the cochlea, we amplified sections of the *Prestin* coding region from pairs of cochleae from single individuals of *Hipposideros armiger*, *Rousettus leschenaulti*, *Miniopterus fuliginosus*, and *Myotis ricketti.* We designed internal primers to sequence sections of the *Prestin* coding gene as follows: exons 3–4 (Fu: 5′-CAG AGT RCY ATG GAT CAT GCT GAA G-3′ and R220: 5′-ACG CTG GCA ACC ACT TAG TTA TG-3′), exons 3–11 (F135: 5′-GCT RAA ACA GGC ATT YAC ATG TAC-3′ and NGSP1: 5′-CTG AAC AAG GCT TCG AGA CAA GGA G-3′), exons 7–16 (GSP2: 5′-GGT GTC TGT AGG TTT GGA TTT GTG GC-3′ and R3: 5′-TCC AGG AAT TTC TTT CAC CTC CTC A-3′), exons 8–10 (NGSP2: 5′-ACC TCA ACG TGT GTT CCC TAG GCG-3′ and Ru: 5′-TCA TTC ACC CTC CAA ATC AAG C-3′) and exons 10–20 (F4: 5′-CCA TAG CCA TCG TTG GAT TTT CAG TG-3′ and Ru: 5′-TCA TTC ACC CTC CAA ATC AAG C-3′). In total we sequenced 220 *Prestin* clones, comprising 67 clones for *Hipposideros* (59 cochlea and 8 brain), 43 for *Rousettus* (30 cochlea and 13 brain), 29 for *Miniopterus* (20 cochlea and 9 brain), and 81 for *Myotis* (62 cochlea and 19 brain).

Our results revealed evidence of multiple splice isoforms in all four species [supporting information (SI) Table S1]. In nearly all cases, isoforms were characterized by one or more missing exons, most commonly exons 8, 9, 10, 12, and 13. Three species also had isoforms with a 52-bp deletion in exon 8 (Table S1). With the exception of a single rare isoform isolated from *Myotis* brain, no missing exons corresponded to the functionally important STAS region (Fig. S2). This finding contrasts with human isoforms, in which the STAS domain was found not to be preserved (1). However, exons 9 and 13, which were found to be missing in several isoforms from both tissue types, did correspond to loop domains. In general, the *Myotis* and *Hipposideros* species showed fewer isoforms with missing exons, as a proportion of clones sequenced, than the *Rousettus* and *Miniopterus*. Nonetheless, based on our results, we conclude that *Prestin* expressed in the cochlea does not differ consistently from that expressed in the brain of the adult bats studied, and, in all four species, isoforms were expressed at lower levels than the complete form in both tissue types.

Phylogenetic Reconstruction. To reconstruct the *Prestin* gene tree we undertook maximum-likelihood (ML) and Bayesian analyses, using the software PAUP* 4.10b (2) and MrBayes 3.1 (3), respectively. For both methods, we used the GTR + I + G nucleotide substitution model selected by MODELTEST 3.6 (4). The ML tree was found by using a heuristic search (tree-bisection-reconnection branch-swapping) with 10 random addition-sequence replicates. ML bootstrap support was obtained from 100 replicates by using a full heuristic search from neighbor-joining starting trees. The same model was used to estimate site-wise likelihood scores also by using PAUP. For the Bayesian analysis, we ran six Metropolis-coupled Markov chains, each with one million generations and a burn-in of 500,000 generations. To test the robustness of the obtained topologies, we also obtained bootstrap values (2,000 replicates) based on neighbor-joining (NJ) and maximum-parsimony (MP) methods in PAUP.

All methods gave broadly consistent topologies with laryngeal echolocating bats forming a monophyletic group, thus conflicting with the established species tree, in which the clade Yinpterochiroptera comprises the nonecholocating Old World fruit bats and some lineages of laryngeal echolocators. To test the confidence of these alternative hypotheses, we derived site-wise log-likelihood values in PAUP for constrained gene and tree topologies, and used these to implement Shimodaira's approximately unbiased test (5) in CONSEL (6). Forced gene and species tree nodes were (*Miniopterus fuliginosus*, *Myotis ricketti*, *Megaderma spasma*, *Rhinolophus ferrumequinum*, *R. luctus*, *R. pusillus*, *Aselliscus stoliczkanus*, *Hipposideros pratti*, *H. armiger*, *H. larvatus*) and (*Rousettus leschenaulti*, *Cynopterus sphinx*, *M. spasma*, *R. ferrumequinum*, *R. luctus*, *R. pusillus*, *A. stoliczkanus*, *H. pratti*, *H. armiger*, *H. larvatus*), respectively.

Fig. 1 Additional Information. Audiograms shown are given for the dog (7), cat (8), human (9), cow (10), horse (10), pig (11), mouse (12), rat (13), rabbit (12), gerbil (14), *R. ferrumequinum* (15), and representatives of the genera *Myotis* (16), *Hipposideros* (17), *Megaderma* (18), *Cynopterus* (19), and *Rousettus* (20). In the case of the *Hipposideros,* the featured species (*H. lankadiva*) calls at around the same frequency as *H. armiger* (65–70 kHz) (17). A mixture of behavioral and neural audiograms is presented.

Although the absolute sensitivities can vary between neural and peripheral audiograms, the overall shape is generally comparable (21). However, because behavioral audiograms often involve testing responses to different tone durations, and quantifying different behavioral responses, audiograms give a reliable measure of auditory sensitivity in relation to frequency within species but comparisons across species must be considered with caution (21).

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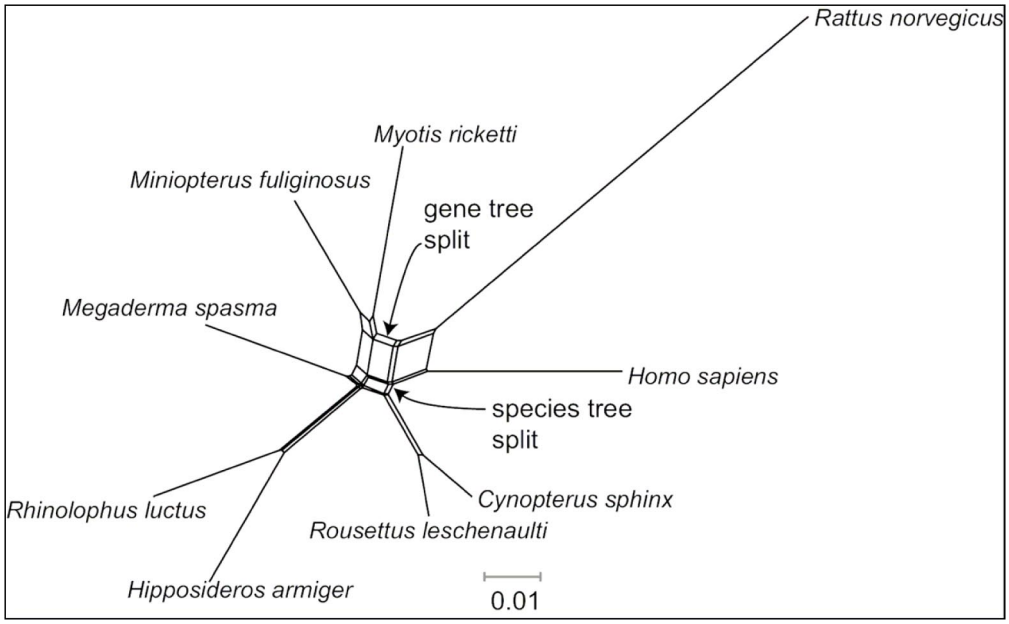
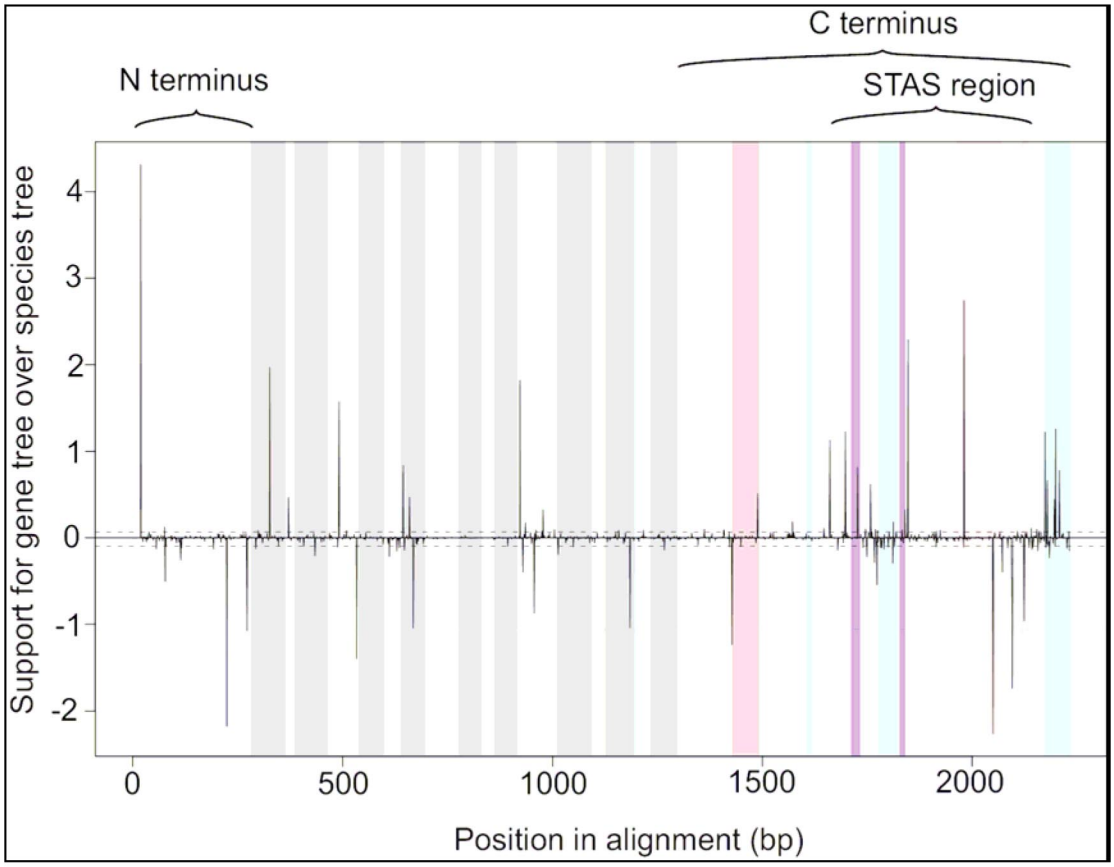


Fig. S1. A neighbor-net network for the *Prestin* gene sequences, showing splits supporting both the maximum-likelihood gene tree and the known species tree, confirming that a subsignal in the data supports the accepted species tree.

(a)



(b)

Fig. S2. Relative support for gene tree and species tree topologies for different sites along the *Prestin* gene sequence (*a*), with corresponding positions of the exons (*b*). In *a*, values are the difference between site-wise negative log likelihood scores for the species tree minus the site-wise negative log likelihood scores for the gene tree. Positive values indicate more support for the gene tree than the species tree. Domains of the prestin protein are indicated: in addition to those labeled on the figure, gray bands indicate transmembrane domains, blue bands coil domains, pink bands α-helices, and purple bands charge clusters. The first α-helix is transmembrane.

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                                                  111 1111111223 3333333344 4444455555 5555555555 5
                     1111111 1222333344 5555789123 4556789250 1126688902 5677900112 2334556667 7
                  7890123457 9267148903 0127581062 6787065248 0400204263 6304779363 6350485685 6
human             NEILAATQRY EIPVRTVPDA FTCIKIGILI GVIGASVFVN KNTTALISTL SRTTLSKKEI YPIINRAVMM R
gerbil            ...PV...KH .....V.SEG .......VF. .......... HS...I.... .......QD. .......L.. .
mouse             ...P.E.... .....V.TEG .......VF. .......... HS...I.... .......QD. ....S..L.. .
rat               ...P.E..KL .....V.T.G ...V...VF. .......... HS...I.... ......TQD. ....S..I.. .
cat               ......S... ..L..KIS.G .......VFV .......... Q......... .......QD. .......L.. K
dog               ....T.S... .....KIS.G .......V.. .......... .......... .......QD. .......... K
rabbit            .GA...A..C .....K.A.G S.Y....V.. ........I. .S......... A......QD. .......... .
cow               ....G..K.. .....K.S.G .......VFV .......... H......... .......QD. .......F.. .
pig               ..L....... .....KIS.G .......VF. .......... .......... .......Q.. .......L.. K
horse             .........S D....K...G .I....RV.. A......... Q......... .......QD. F...S..H.. .
Rousettus leschenaulti ..... .....KI..G .......VF. .......... H........M .......QD. .......F.. .
Cynopterus sphinx .........K. .....KI..G .......VF. .......... H........M ....M..QD. .S.....L.. .
Myotis ricketti   T.....A.K. D.LAI.I..G .......VF. .....A...S H.S.G..A.M ....M..QD. ....S..F.. K
Miniopterus fuliginosus T........C DLLA.KI..G .......VF. ....I....S H........M ....M..QD. ...VS..L.. K
Megaderma spasma  T.......K. ..S..KIS.G ....N..VF. .........S H........M ....M..QD. ..V.S.TL.. K
Rhinolophus luctus T.L...AE.. ..L..KIS.G ....N..VFV .AV.FAI..G H.....TA.M .K.SMTTQD. .S..S..FL. K
Rhinolophus ferrumequinum T......E.. ..L..KIS.G ....N..VF. .AV.FA...G H.....TAIM ..VSMTTQD. ......SFL. K
Rhinolophus pusillus T......E.. ..L..KIS.G ....N..VF. .AV.FA...G H.....TA.M ...SMTTQD. ....S.SFL. K
Hipposideros pratti T...E..E.. ..LA.KIS.G ....NV.VF. .APSFA.A.. H..S..TA.M ...SMTTQD. ......SFLI .
Hipposideros larvatus T...E..E.. ..LA.KIS.G ....NV.VF. .APSFA.A.. H..S..TA.M ...SMTTQD. ......SFLI .
Hipposideros armiger T...E..E.. ..LA.KIS.G ....NV.VF. .APSFA.A.. H..S..TA.M ...SMTTQD. ......SFLI .
Aselliscus stoliczkanus T...E..E.. ..LA.KIS.G ....NV.VF. .APSFA.A.. H..S..TA.M ...SMTTQDK ....STSFLI K

                  5555555555 5666666666 6666666666 6666666666 6666666677 7777777777 7777777777
                  7888899999 9000000011 1111222222 3333334566 6688889900 0011122222 2222333444
                  7257812357 8123457901 2469123459 3567892201 2314591901 3445902345 6789139034
human             KVAMATVVAA EGEDATPEED GVYIIKSTFM MPGDNVVILA GIAVNRRWEL FHQLLAQEAS APPSELPAEA
gerbil            ...I.A..VG ...N...... D.....T..L .QTE..I... VMP....K.. ..HVM..... ...PDM.T..
mouse             ...V....V. ...N...... D.F...T..L LQ.E...V.. ..P...NK.. ...VM....T .SLP.M.T..
rat               ...I....V. ...N...... D.F...T..L LQ.E.I.M.. .....S.K.. ...VM...TT VL.P.M.T..
cat               ......A.V. .......... D......... .....II... .....Q.K.. .Y.......T .A.P.S--..
dog               E..V....V. .......Q.. D........L ....TI.... .....Q.K.. .......... .L.P.S.T..
rabbit            ...I...AV. ...NTP...E E...VR...L ....G.I.V. .......K.. .Y.VM....L .A.A.M.T..
cow               ........V. ....G...DE NI......LL .....II... .......L.. ...A....G. .V.P.S--..
pig               .M...A..V. ....G...GE DI.....K.L .....II... VM..D..R.. ...VM.RD.. ...L.S--..
horse             ........V. A...G....E DIFVT....L .....II... .....Q.KK. ...V...... ...P.S--..
Rousettus leschenaulti .....A.I.. ....G....- D.....N..L .....I.... .....QQR.. ..HV...... ..TT..--..
Cynopterus sphinx .....A.... ....G....- D.....N..L .....I.... .....QQR.. ..IV...... ..TT..--..
Myotis ricketti   ...L..II.G ......T... DIFV..T.IL .........S .....SFK.. .........L T..P.AQ-..
Miniopterus fuliginosus ......I..G ........D. EIF......L .........S .....QLK.. .........L T..P.S--..
Megaderma spasma  ....V..... ..G.G..... DIF.T....L .....I...S ..P..QQL.. ...V.....L ...P.S--.P
Rhinolophus luctus .GII..D... .A..G....G D..V.....L ..LQ.I...Q ...ISQ.LD. ..LV.EK.VA .TMP.S--DV
Rhinolophus ferrumequinum .GII..D... .A..G....E D..V.....L ..LE..I..Q ....S..LD. ..LV.EK..A .ATP.S--DV
Rhinolophus pusillus .GII..D... .A..G.A..E D..VT....L ..LE.....Q ...IS.QLD. Y.LV.EK..A .ATP.S--DV
Hipposideros pratti .GII.VD.V. .A..G....E EI.V.....L ..LE...... ....S.QL.. ..LV.EK..A VTTL.S--.V
Hipposideros larvatus .GII.ID... .A..G....E EI.V.....L ..LE...... ....S.QL.M ..LV.EK..A VTTL.S--.V
Hipposideros armiger .GII.VD... .A..G....E EI.V.....L ..LE...... ....S.QL.. ..LV.EK..A VTTL.S--.V
Aselliscus stoliczkanus .GII.VD... .A..G....E EI..T...LL ..LE...... ....S.QL.. ..LV.EK..A .TTP.S--.V
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Fig. S3. Alignment of the amino acid sequences of the *Prestin* gene from 22 mammals (only the variable sites are shown).

Table S1. Parameter estimates for *Prestin* data for various codon-based models of nucleotide sequence evolution

Branch-site model	ℓ	Parameters	Positively selected sites
Model A (null hypothesis) for CF bats	−11417.23	$P_0 = 0.766$, $P_1 = 0.079$, $P_{2a} = 0.141$, $P_{2b} = 0.015$ Background: $\omega_0 = 0.021$, $\omega_1 = 1.00$, $\omega_{2a} = 0.021$, $\omega_{2b} = 1.00$ Foreground: $\omega_0 = 0.021$, $\omega_1 = 1.00$, $\omega_{2a} = 1.00$, $\omega_{2b} = 1.00$	None identified
Model A (alternative hypothesis) for CF bats	−11411.51	$P_0 = 0.876$, $P_1 = 0.088$, $P_{2a} = 0.033$, $P_{2b} = 0.003$ Background: $\omega_0 = 0.022$, $\omega_1 = 1.00$, $\omega_{2a} = 0.022$, $\omega_{2b} = 1.00$ Foreground: $\omega_0 = 0.022$, $\omega_1 = 1.00$, $\omega_{2a} = 13.791$, $\omega_{2b} = 13.791$	14E, *157A*, 158V, **170F**, 186A, *384T*, 392A, *474S*, *507T*, *509T*, 565S, *568L*, *582G*, **585I**, 592D*601A*, *636L*, 637E, *685S*, 714L, **720E**, 722K, *744V*
Model A (null hypothesis) for fruit bats	−11430.86	$P_0 = 0.901$, $P_1 = 0.099$, $P_{2a} = 0.00$, $P_{2b} = 0.00$ Background: $\omega_0 = 0.023$, $\omega_1 = 1.00$, $\omega_{2a} = 0.023$, $\omega_{2b} = 1.00$ Foreground: $\omega_0 = 0.023$, $\omega_1 = 1.00$, $\omega_{2a} = 1$, $\omega_{2b} = 1$	None identified
Model A (alternative hypothesis) for fruit bats	−11430.86	$P_0 = 0.901$, $P_1 = 0.1$, $P_{2a} = 0.00$, $P_{2b} = 0.00$ Background: $\omega_0 = 0.023$, $\omega_1 = 1.00$, $\omega_{2a} = 0.023$, $\omega_{2b} = 1.00$ Foreground: $\omega_0 = 0.023$, $\omega_1 = 1.00$, $\omega_{2a} = 1$, $\omega_{2b} = 1$	7T
Model A (null hypothesis) for Yangochiroptera	−11430.86	$P_0 = 0.9$, $P_1 = 0.099$, $P_{2a} = 0.001$, $P_{2b} = 0.00$ Background: $\omega_0 = 0.023$, $\omega_1 = 1.00$, $\omega_{2a} = 0.023$ $\omega_{2b} = 1.00$ Foreground: $\omega_0 = 0.023$, $\omega_1 = 1.00$, $\omega_{2a} = 1$, $\omega_{2b} = 1$	None identified
Model A (alternative hypothesis) for Yangochiroptera	−11430.78	$P_0 = 0.901$, $P_1 = 0.099$, $P_{2a} = 0.001$, $P_{2b} = 0.00$ Background: $\omega_0 = 0.023$, $\omega_1 = 1.00$, $\omega_{2a} = 0.023$, $\omega_{2b} = 1.00$ Foreground: $\omega_0 = 0.023$, $\omega_1 = 1.00$, $\omega_{2a} = 329.147$, $\omega_{2b} = 329.147$	19E, 27V, 597A

Sites potentially under positive selection, using *Rhinolophus ferrumequinum* as a reference, are shown. Posterior probability values (*P*) between 0.5 and 0.8 are in plain text, between 0.8 and 0.95 in italics, and greater than 0.95 in boldface type.

Table S2. Splice variants isolated and amplified from brain (gray) and cochlea (blue) tissue. The box in each row denotes the expected product based on the primers used. The asterisk denotes the location of a 52-bp deletion.