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Report

Cetaceans on a Molecular Fast Track to Ultrasonic Hearing

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Summary

The early radiation of cetaceans coincides with the origin of their defining ecological and sensory differences [1, 2]. Toothed whales (Odontoceti) evolved echolocation for hunting 36–34 million years ago, whereas baleen whales (Mysticeti) evolved filter feeding and do not echolocate [2]. Echolocation in toothed whales demands exceptional high-frequency hearing [3], and both echolocation and ultrasonic hearing have also evolved independently in bats [4, 5]. The motor protein Prestin that drives the electromotility of the outer hair cells (OHCs) is likely to be especially important in ultrasonic hearing, because it is the vibratory response of OHC to incoming sound waves that confers the enhanced sensitivity and selectivity of the mammalian auditory system [6, 7]. Prestin underwent adaptive change early in mammal evolution [8] and also shows sequence convergence between bats and dolphins [9, 10], as well as within bats [11]. Focusing on whales, we show for the first time that the extent of protein evolution in Prestin can be linked directly to the evolution of high-frequency hearing. Moreover, we find that independent cases of sequence convergence in mammals have involved numerous identical amino acid site replacements. Our findings shed new light on the importance of Prestin in the evolution of mammalian hearing.

Results

Our analyses of an extended data set of *Prestin* sequences showed that amino acid replacements in the evolution of cetaceans have coincided with increases in the frequency of their vocalizations and the associated increased auditory sensitivity at higher frequencies. Molecular adaptation occurred in two episodes, one on the ancestral branch of all echolocating toothed whales and the second on the ancestral branch of the crown group of small toothed whales, which are among the highest-frequency echolocators. Convergence with echolocating bats is extensive and has involved ten amino acid replacements that also define *Prestin* convergence among lineages of echolocating bat.

Phylogenetic Reconstruction

We combined new and published *Prestin* sequences [9] to obtain coverage of the major cetacean lineages [2, 12]. Our new Bayesian tree confirmed the earlier reported convergence between bats that use constant frequency (CF bats) echolocation (horseshoe and leaf-nosed bats) and dolphins [see 9, 10] (Figure 1). However, CF bats now moved into the cetacean clade rather than vice versa [9] as a result of our increased support for cetacean monophyly. We found high support for a clade of toothed whales and horseshoe bats (posterior probability 0.9) but weaker support for horseshoe bats as sister group to the small whales (Delphinidae, Phocoenidae, and Ziphiidae; posterior probability 0.5), though the level of support for the convergent relationships varied depending on the details of the phylogenetic analysis used, as expected given the known complex pattern of molecular evolution in these data. Our Bayesian phylogeny also supports the previously reported convergence between other echolocating bats [11]. We refer to convergent molecular evolution between echolocating bats and cetacean lineages as "bat-whale convergence" and among different clades of echolocating bats as "bat-bat convergence."

Identifying Convergent and Divergent Sites

Figure 2A shows the total posterior probability of divergent and convergent substitutions for all pairs of internal branches from our Bayesian analysis of convergent substitutions. At a 5% critical value, three of our twelve bat-whale convergence focal branch pairs and two of our three focal branch pairs for putative bat-bat convergence showed significantly higher probabilities of convergence than expected under our neutral null substitution model (Figure 2B). Thus, convergent molecular evolution has occurred between a number of different sets of branch pairs, and neither bat-whale nor bat-bat convergent evolution is localized to a particular bat lineage.

Our analysis also allows us to identify sites likely to be involved in convergent changes. Figure S1 (available online) shows the distribution of sites with a > 0.5 posterior probability of showing either bat-whale or bat-bat convergent substitutions. A test of association between counts of convergent sites and protein domain [13] revealed nonrandom distributions along the gene in the case of both bat-whale convergence (χ^2 = 12.378, degrees of freedom [df] = 4, p = 0.01475) and bat-bat convergence (χ^2 = 21.96, df = 4, p = 0.0002). These results were also confirmed by Fisher's exact test based on simulation (for bat-whale sites, p = 0.01694; for bat-bat sites, p = 0.000284; based on 10^6 replicates in each case). In both cases of convergence, the nonrandom distribution of convergent sites was driven by significant over-representation in the cytoplasmic termini of the protein. Specifically, the probability that sites in the cytoplasmic C and N termini of the protein show convergence was greater than that for sites in the rest of the gene (odds ratio [OR] = 3.23 for bat-whale convergent sites, 95% confidence interval [CI] for OR: 1.34–7.78; OR = 5.72 for bat-bat convergent sites, 95% CI: 2.16–15.15). Furthermore, we found that in both cases of convergence the STAS domain was 2-fold enriched over the rest of the cytoplasmic part of the protein, though this was not statistically significant (OR = 2.18 for bat-whale convergent sites, 95% CI: 0.68–6.97; OR = 2.16 for bat-bat convergent sites, 95% CI: 0.75–6.16).

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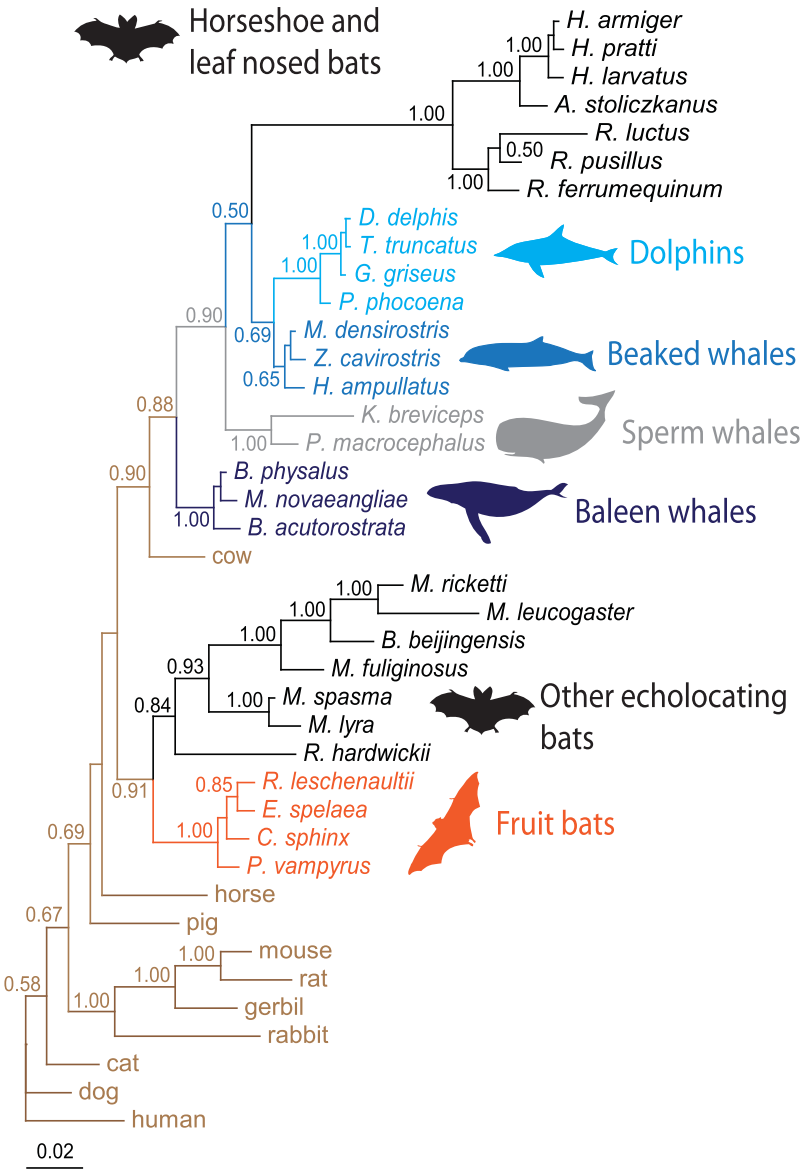

Figure 1. Bayesian Phylogeny of Prestin

Phylogeny of Prestin protein sequences, under the amino acid GTR model as implemented in MrBayes. Posterior probabilities larger than 0.5 are shown on the nodes.

Out of a total of 26 convergent substitutions implicated in bat-whale convergence and 29 in bat-bat convergence, ten amino acid substitutions were common to both cases (Figure S1 available online). Thus, the two independent cases of convergence have involved multiple parallel amino acid substitutions, though there are also many convergent substitutions unique to each case. Too few sites were identified as being convergent in both cases to allow meaningful statistical analysis of their distribution along the gene.

Molecular Evolution of Prestin in Cetaceans

Figure 3A shows the amino acid substitutions that have occurred during the evolution of Prestin in whales. Ancestral sequences were estimated on the basis of the free-ratio model in CODEML (log-likelihood ratio test = 294.88, df = 75, $p < 0.001$). Immediately apparent is the faster rate of protein evolution in the toothed whales (56 substitutions) in comparison to baleen whales (11 substitutions), consistent with most changes in cetaceans being adaptations to increased auditory sensitivity at high frequencies for echolocation. Many of the amino acid changes (34 out of a total 56) in toothed whales are convergent with substitutions found in echolocating bats. The largest number of amino acid changes on any single lineage happened after the split between pygmy sperm whale and sperm whale, which includes changes at several amino acid sites where the pygmy sperm whale shows convergence with echolocating bats that are not convergent in other cetaceans. An equivalent species tree of bats with mapped substitutions also shows many more substitutions in both lineages of echolocating taxa in comparison to nonecholocating fruit bats (Figure S2A).

Prestin Evolution and High-Frequency Hearing

We found a significant association between the number of nonsynonymous substitutions leading to each cetacean species and the estimated frequency of best hearing sensitivity for that species ($p = 0.0022$, Figure 3B), but no correlation between such hearing sensitivity and the number of synonymous changes ($p = 0.143$). The correlation between nonsynonymous changes and frequency remained significant after correcting for phylogeny by using an independent contrasts test ($p = 0.0308$, Figure 3C). The same analysis was repeated for bats, in which a significant correlation between hearing frequency and total nonsynonymous changes ($p = 0.0067$; for synonymous changes, $p = 0.7806$) was no longer significant after a phylogenetic correction was applied ($p = 0.6811$, Figures S2B and S2C). These results strongly implicate changes in the Prestin protein in the evolution of increased hearing sensitivity at higher frequencies in echolocating whales and dolphins.

Inferring Selective Regime

Our stepwise procedure confirmed that the selective pressure on Prestin in all five branches examined different significantly from a "background" d_N/d_S ratio (ω) measured across the rest of the tree (Table S1). In every case, ω values were higher than background values, and in two cases (branches ancestral to toothed whales and to the small whales clade) were greater than 1 (2.515 and 2.571, respectively, see Figure 2B). For the other branches examined, the ω value for the ancestral branches of dolphins was 0.431, that of the ancestral pygmy sperm whale branch was 0.703, and that of the ancestral horseshoe bats branch was 0.754. None of these values was significantly different from 1 ($p > 0.05$ in each case). Although we cannot reject a null hypothesis of neutral evolution for any of the branches tested and therefore these elevated ω values could be explained by a relaxation of purifying selection in these ancestral branches, we believe that these results,

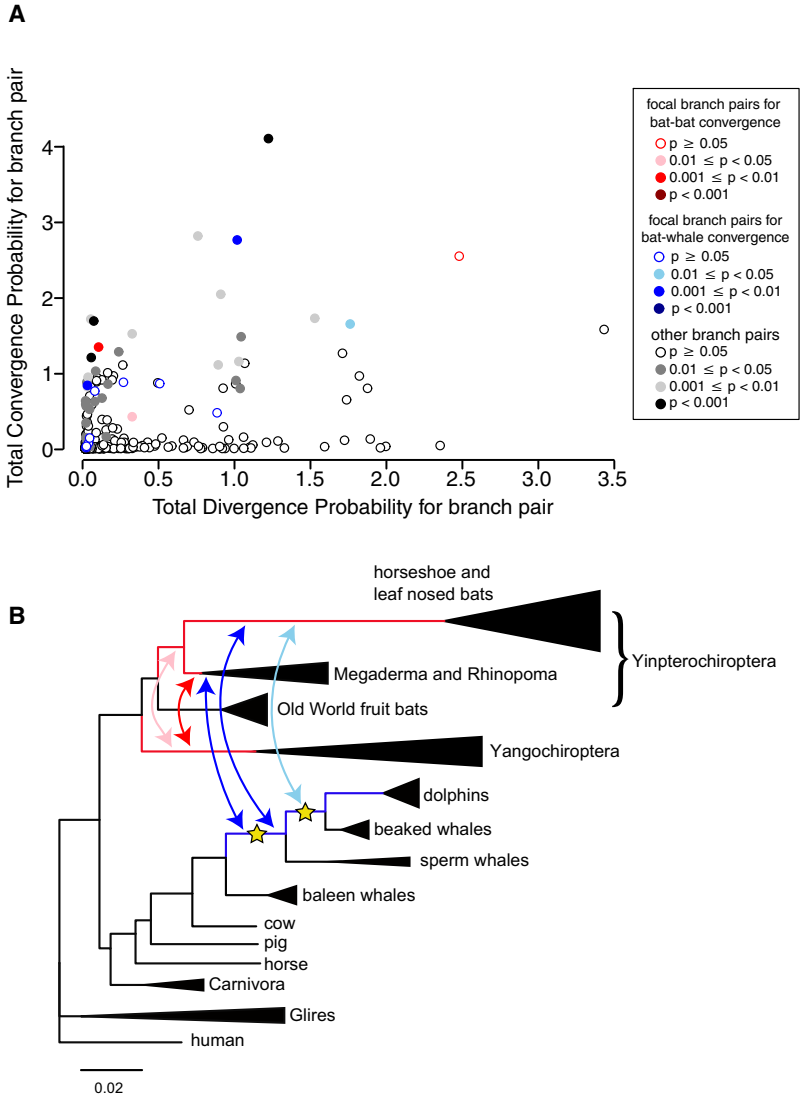

Figure 2. Sequence Convergence in the *Prestin* Gene among Echolocating Dolphins and Bats

(A) Convergence and divergence support for pairs of branches (Bayesian posterior probability). The three focal branch pairs of ancestral echolocating bat are shown in shades of red, the 12 focal branch pairs of echolocating bats and whales in shades of blue (horseshoe + leaf-nosed bats versus toothed whales denoted by the upper dark blue point, *Megaderma* + *Rhinopoma* versus toothed whales by the lower dark blue point), and all other pairs in shades of gray. In each case, shade intensity corresponds to the probability of observing the shown convergence level by chance under a standard substitution model.

(B) Simplified species tree, showing branch pairs identified as significantly convergent. Four red lineages are focal branches of ancestral echolocating bat, and three blue ones are focal branches of echolocating whale. Two yellow stars indicate that those branches are identified as being under positive selection.

taken together, provide fairly strong evidence that positive selection has driven the evolution of Prestin to allow high-frequency hearing sensitivity in echolocating cetaceans. Our reasoning is (1) the elevated ω in all ancestral branches leading to high-frequency hearing in cetaceans, (2) the association between high ω and branches showing bat-whale convergent substitutions, and (3) the known importance of Prestin in high-frequency hearing, including our results demonstrating an association between amino acid substitutions in Prestin and inferred hearing-frequency sensitivities. Moreover, the importance of high-frequency hearing for successful echolocation makes the action of positive selection highly plausible in this case.

Discussion

We combined new data with in-depth statistical analyses to dissect and characterize the nature and extent of sequence convergence in the *Prestin* gene among several lineages of echolocating taxa. Our new results reveal that Prestin has a much more complex evolutionary history in echolocating mammals than was previously realized.

Tests of branchwise convergence among ancestral branches of echolocating bats and whales revealed that five pairs of branches were characterized by significantly high sequence convergence, three between echolocating bats and toothed whales and two between echolocating bats. Nonetheless, convergent sites were not restricted to these branch pairs but also occurred between other branches in both groups. Examining all branch combinations revealed a total of 26 sites supporting bat-whale convergence and 29 supporting bat-bat convergence. Remarkably, ten of these sites were common to both independent cases, in contrast to only three shared sites that Li et al. [10] implicate in driving the phylogenetic misplacement of the dolphin and two microbats in their much smaller data set.

We found that *Prestin* has in fact undergone two clear episodes of accelerated protein evolution in cetaceans: on the ancestral branch of all toothed whales and on the branch leading to the dolphins plus beaked whales. Several lines of evidence support the idea that these episodes represent bursts of adaptive change. Closer inspection of the latter episode showed that the dolphins have undergone numerous subsequent amino acid changes during their radiation, whereas there have been relatively few changes in the clade of beaked whales, which instead are characterized by strong purifying selection. These results indicate that *Prestin* has likely had an important role in the evolution of increased sensitivity of toothed whales to high-frequency sound; thus, they also support the assertion that echolocation evolved in toothed whales after they had split from baleen whales [2] rather than being an ancestral characteristic of all cetaceans.

Comparing analyses of selection and convergence showed that both bursts of selection in the cetaceans have involved multiple amino acid substitutions, including sites implicated in bat-whale convergence (five and four sites, respectively) and sites implicated in bat-bat convergence (two sites in each branch). Thus, similar sets of sites have driven two different examples of convergence in the *Prestin* gene of echolocating taxa, suggesting functional importance. Given the lack of empirical audiogram data for mysticetes and some

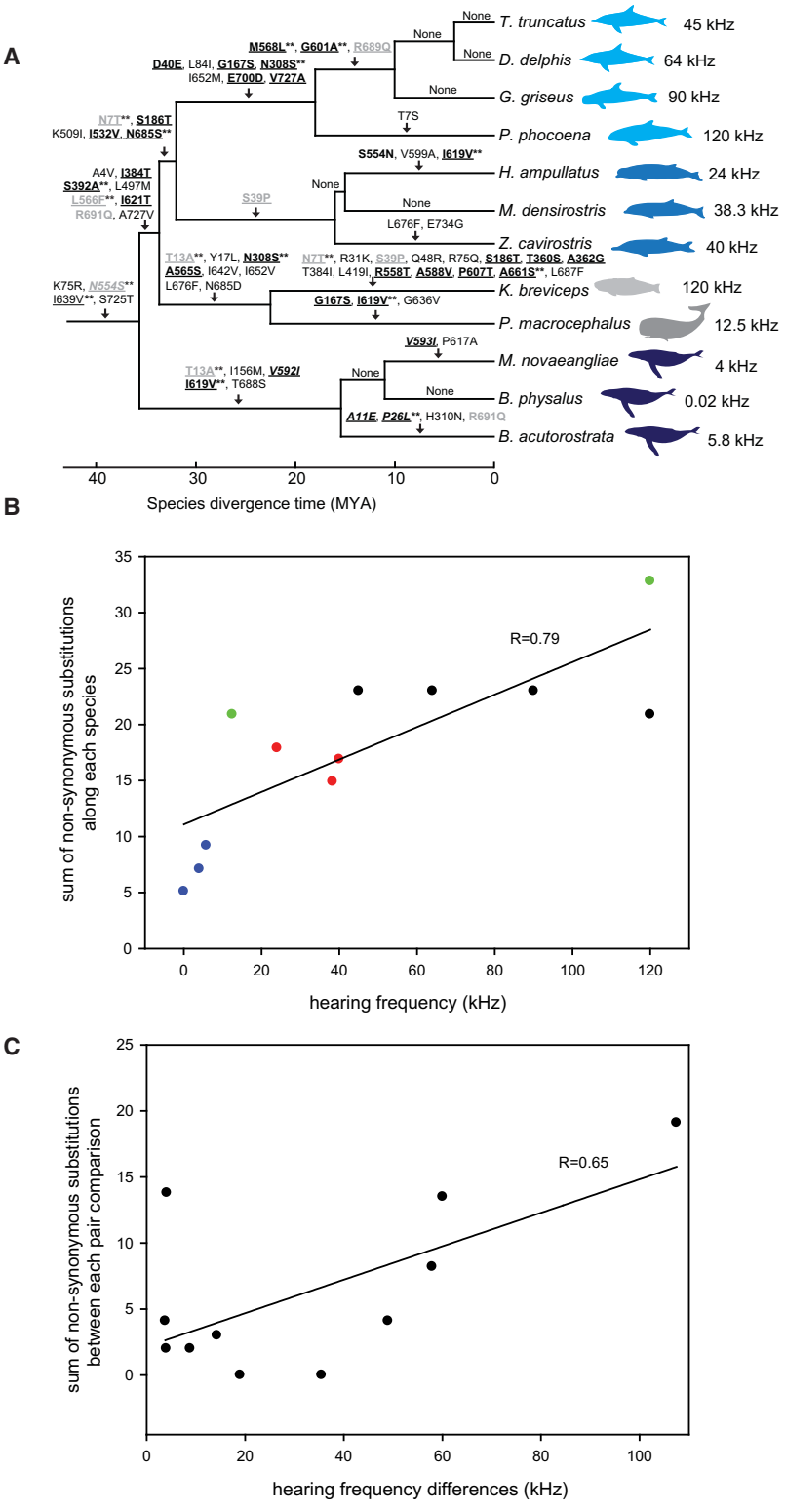

Figure 3. Association between Evolution of the *Prestin* Gene and the Evolution of High-Frequency Hearing in Cetaceans

(A) Amino acid changes mapped onto the cetacean phylogeny. Bold indicates sites that are convergent with echolocating bats on the basis of parsimony, and underlining indicates sites that are convergent with echolocating bats on the basis of Bayesian inference (posterior probability > 0.5). Two asterisks indicate sites that are common to both bat-whale and bat-bat convergence. Italics indicate sites that are convergent between echolocating bats and either the ancestral branch of all whales or any branch within the clade of baleen whales. Gray indicates sites that are unresolved because of uncertain ancestral states. Divergence times shown are means from ref. 1.

(B) Plot of estimated frequency of best hearing sensitivity versus the number of nonsynonymous substitutions in whales, showing a significant relationship (R = 0.79, p = 0.0022). Dolphins are shown as black circles, beaked whales as red circles, sperm whales as green circles, and baleen whales as blue circles.

(C) Independent contrasts of the number of nonsynonymous substitutions in whales versus corresponding differences in the estimated frequency of best hearing sensitivity (R = 0.65, p = 0.0308, respectively).

See Figure S2 as well.

odontocetes, we also used published call data to infer the frequency of best hearing sensitivity, assuming that their ears are most highly tuned to their own calls (and those of their conspecifics). Although this method is not ideal, particularly if our assumption is unsafe, we found a significant association between the number of amino acid replacements between sister taxa and the difference in their inferred best hearing frequencies (Figure 3). Most strikingly, the single largest number of amino acid replacements occurred on the branch leading to the pygmy sperm whale (*Kogia breviceps*), which echolocates at much higher frequencies than its larger sister species, the sperm whale [14, 15].

Some support for a close link between amino acid replacements (including convergent ones) and hearing was also suggested from our analysis of bats, though the correlation between estimated hearing sensitivity and number of replacements was not significant after phylogenetic correction. Like whales, however, hearing-frequency values could often only be estimated from spectrograms, rather than from audiograms, thus possibly limiting the power of this analysis and potentially even obscuring a real association between actual hearing frequency and Prestin evolution. Nonetheless, it is interesting that the amino acid changes in echolocating whales appear to have followed an evolutionary trajectory that has more in common with that of horseshoe bats than of other echolocating bats. This is intriguing because horseshoe bats and their allies have among the highest echolocation call frequencies [16] and the associated highest-frequency hearing abilities [17, 18] of species with similar body mass. Furthermore, these bats were previously showed to have undergone positive selection at the *Prestin* gene around 52 to 39 million years ago [11, 19]. By comparison, our data support the idea that echolocation and high-frequency hearing in cetaceans are more recent evolutionary innovations and occurred rapidly, with both episodes of adaptive convergence happening within a period of about five million years, around

31 million years ago (see ref. 1). Thus, the echolocating cetaceans, especially dolphins, can be seen as having taken a molecular fast track to sensitive high-frequency hearing. Regardless of the timing, the correlation between nonsynonymous changes and inferred hearing-frequency ability indicates that several keys sites in *Prestin* are likely to be especially important in the tuning of sensitivity to high-frequency sound. This finding hints at parallels with the so-called critical sites of visual opsin pigments, which have been shown to have an additive effect on peak spectral sensitivity [20]. Expression of recombinant Prestin proteins would help to test this prediction [20], and "high-frequency *Prestin*" gene knockin of mice, combined with measurements of otoacoustic emissions, has enormous potential for improving our understanding of the molecular basis of hearing.

The extent and nature of Prestin protein convergence among echolocating mammals is all the more remarkable given the scarcity of other known examples. Although several recent papers have reported convergent amino acid replacements, these have tended to involve small numbers of substitutions associated with particular ecological and morphological traits [21–25, reviewed in 26]. More extensive sequence convergence was discovered in the mtDNA genes of some reptiles, though the adaptive significance of this convergence is unclear [27]. Arguably, the example of convergence most similar to Prestin in terms of magnitude and recurrence is the enzyme lysozyme, which shows similarities in ruminants, primates, and birds that have evolved foregut fermentation [28–30]. Three independent origins of digestive lysozyme in foregut fermenters (hoatzin-cow-monkey) have involved the same two sites [31], far fewer than the ten parallel convergent sites we find in *Prestin*.

Accession Numbers

The GenBank accession numbers for the new *Prestin* sequences reported in this paper are HQ176004–HQ176008.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, two figures, and three tables and can be found with this article online at doi:10.1016/j.cub.2010.09.008.

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