The hearing gene *Prestin* unites echolocating bats and whales

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Echolocation is a sensory mechanism for locating, ranging and identifying objects which involves the emission of calls into the environment and listening to the echoes returning from objects [1]. Only microbats and toothed whales have acquired sophisticated echolocation, indispensable for their orientation and foraging [1]. Although the bat and whale biosonars originated independently and differ substantially in many aspects [2], we here report the surprising finding that the bottlenose dolphin, a toothed whale, is clustered with microbats in the gene tree constructed using protein sequences encoded by the hearing gene *Prestin*.

Mammalian prestin is a member of the SLC26 anion-transport family found primarily on the membrane of cochlear outer hair cells (OHCs). It is composed of intracellular amino and carblyx termini, about ten transmembrane domains, and multiple intracellular and extracellular loops [3]. Prestin provides the electromobility of OHCs that is thought to be responsible for cochlear amplification, an active process that confers sensitivity and frequency selectivity to the mammalian auditory system [4]. To examine the potential role of prestin in echolocation, we compiled all eutherian *Prestin* sequences that are publicly available. The resulting sequence data of 25 species include ten (echolocating) microbats and three (nonecholocating) megabats, but unfortunately only one toothed whale, for which the *Prestin* sequence is incomplete. We thus amplified and sequenced all 18 coding exons of *Prestin* from the genomic DNA of a bottlenose dolphin (*Tursiops truncatus*) and used this sequence in subsequent analysis.

Using maximum likelihood (ML), maximum parsimony (MP), and neighbor-joining (NJ) methods [5], we reconstructed the prestin protein tree. Surprisingly, all methods group the dolphin within the bats — specifically with the green-labeled families of Rhinolophidae and Hipposideridae in Figure 1A — rather than with the cow, its true closest relative in our data, and this unexpected grouping has a significant bootstrap support (Figure 1A). Furthermore, as recently reported [6], unlike the species tree where microbats are paraphyletic [7] (Figure 1B), the prestin tree clusters the ten microbats in exclusion of the three megabats with a moderate bootstrap support, resulting in the misplacement of two purple-labeled microbats (Figure 1A,B). Although the prestin tree also has other differences from the mammalian species tree, none of these differences are statistically supported (Figure 1A).

What could have caused the misplacement of dolphin to the bat clade in the prestin tree? Horizontal gene transfer, DNA contamination, gene paralogy, long-branch attraction, and biased amino acid frequencies are all unlikely (see Supplemental Experimental Procedures and Figure S1A in the Supplemental Data). The only remaining reason is the convergence of the prestin sequences of echolocating bats and whales, likely resulting from a common selection for amino-acid-altering mutations that are beneficial to echolocation. Indeed, the same misplacement of dolphin is observed in the *Prestin* tree reconstructed with only nonsynonymous nucleotide substitutions (Figure S1B); but, when only synonymous substitutions are used, dolphin and cow are correctly grouped with 100% bootstrap support (Figure S1C).

We used two approaches to identify the amino-acid sites causing the clustering of dolphin and bat prestins. To avoid confounding factors, we analysed a subset of 18 species that includes only cetartiodactyls and bats, with human and mouse as outgroups (Figure 1B). The prestin protein tree again places dolphin within bats and groups all echolocating bats (Figure S2). We found that a minimum of nine amino acid sites (referred to as set I in Figure 1C) had to be removed to make the likelihood of the species tree higher than that of the prestin tree (see Supplemental Experimental Procedures). Because the prestin tree misplaces both dolphin and two purple-labeled microbats (Figure 1A), we further examined whether the two problems are caused by the same sites. We found that a minimum of seven sites (referred to as set II in Figure 1C) had to be removed to correct the phylogenetic position of dolphin, and a minimum of five sites (referred to as set III in Figure 1C) had to be removed to rectify the positions of the two microbats. Sets II and III share three sites, significantly exceeding the random expectation ($P < 0.002$, binomial test), suggesting that the misplacements of the dolphin and the two microbats are in large part due to the same sites.

Our second approach was to identify convergent or parallel amino acid substitutions [8] that occurred between the dolphin branch (red in Figure 1B) and any of the three bat branches marked 1, 2, and 3 in the established species tree in Figure 1B, by comparing the inferred ancestral prestin sequences in all interior nodes of the species tree and the extant sequences. We considered these three bat branches because prestin function associated with echolocation in bats (especially Rhinolophidae and Hipposideridae) likely emerged in one or more of these branches. We identified no convergent site, but four, one and six parallel sites between the red branch and branches 1, 2, and 3, respectively, and named these three sets of sites as sets 1, 2, and 3, respectively (Figure 1C). A statistical test [8] shows that sets 1, 2, and 3 contain significantly more parallel sites than their respective random expectations ($P < 10^{-8}$, < 0.0045, and $< 10^{-8}$, respectively), suggesting that a common selection, rather than chance, underlies the observed parallel substitutions. This is the largest number of parallel substitutions observed in any proteins inferred to have undergone parallel sequence evolution [8–10]. Six of the seven sites in set II, which cause the misplacement of dolphin, experienced parallel changes (Figure 1C), supporting our hypothesis that parallel amino acid substitutions is the reason for the grouping of dolphin and bats in the prestin tree.

After mapping the sites of sets I–III and sets 1–3 onto the structural model of prestin [3] (Figure 1C), we observed that all except three sites fall in the intracellular terminal regions, including one site in the amino terminus and ten in the carboxyl terminus, a pattern that is highly nonrandom ($P < 0.005$, Fisher's exact test). Previous mutagenesis studies demonstrated that both amino and carboxyl termini are used for voltage sensing [3] and

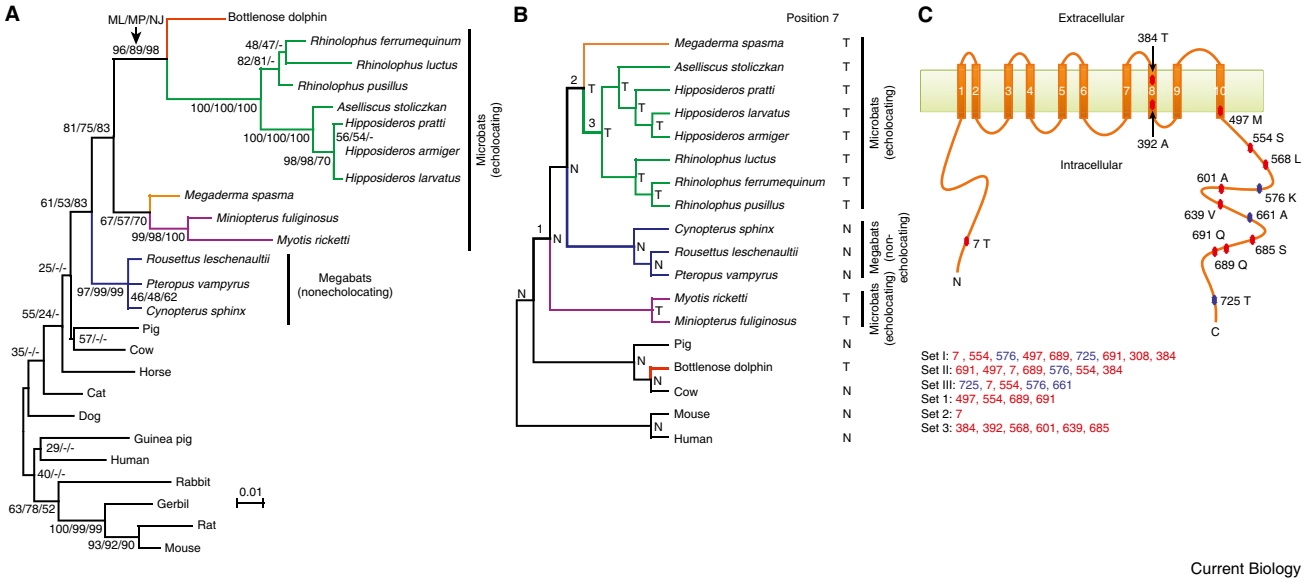

Figure 1. Parallel evolution of prestins of echolocating bats and bottlenose dolphin.

(A) The maximum-likelihood (ML) tree reconstructed using the prestin protein sequences of 25 mammals, under the model of JTT-f with a gamma distribution of substitution rate variation among sites (shape parameter = 0.14). Numbers on interior branches are bootstrap percentages from ML, maximum-parsimony (MP), and neighbor-joining (NJ) analyses. The bootstrap value from an analysis is indicted as "-" when the branch does not exist in that analysis. (B) The species tree of 18 mammals. Tree branches are not drawn to scale. Parallel substitutions were examined between the red branch and the branches labeled 1, 2 and 3. The amino acid (N: Asn; T: Thr) at position 7 of prestin is shown for each interior and exterior node. (C) Locations of evolutionarily interesting amino acid sites in the structural model of prestin with ten transmembrane domains. Numbers associated with colored circles are the amino acid positions in the dolphin prestin sequence, with the residues observed in dolphin indicated. Sets I, II, and III are the most important sites responsible for the misplacement of both dolphin and the two purple-labeled microbats in panel A, misplacement of dolphin, and misplacement of the two microbats, respectively, and are listed in the order of their support of the gene tree relative to the species tree (from high to low). Sets 1, 2, and 3 are sites that have experienced parallel amino acid substitutions between the dolphin branch (red in panel B) and branches 1, 2, and 3, respectively. Parallel evolution sites are shown in red, while the other sites are shown in blue.

the amino terminus is also critical for homo-oligomerization of prestin [3], which may influence the speed of conformational changes of prestin that is likely crucial for high-frequency acoustic sensitivities of echolocation. Although not all parallel changes identified may be necessary for echolocation, as some have reverted in some microbats or occurred also in some nonecholocating mammals, they are strong candidates for future experimental investigation. Of particular interest is position 7, which appears in sets I–III and experienced a parallel change from Asn to Thr in three branches (Figure 1B). This site has a Thr in all echolocating mammals but an Asn in all nonecholocating mammals examined so far. We were able to amplify exon 1 of *Prestin* from the bowhead whale *Balaena mysticetus*, a nonecholocating whale, and determined that it has an Asn at position 7. Thus, the multiple Asn to Thr changes at position 7 were likely important for the multiple origins of echolocation. Sequencing *Prestin* from additional echolocating and nonecholocating cetaceans will further help identify the amino acid changes critical for echolocation.

Our findings suggest that the high-frequency acoustic sensitivities and selectivities of bat and whale echolocation rely on a common molecular design of prestin. Because prestin function can be studied in knock-in mice and in cell lines [4], a functional analysis of the parallel amino acid substitutions identified here could shed light on the structure-function relationship of prestin and the molecular underpinnings of the acoustic adaptations in echolocation. It could also help answer why the prestin of bottlenose dolphin is particularly similar to that of Rhinolophidae and Hipposideridae bats (Figure 1A).

Supplemental Data

Supplemental data are available at http://www.cell.com/current-biology/supplemental/S0960-9822(09)02057-0

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