Functional Effects of a Retained Ancestral Polymorphism in *Prestin*

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Abstract

Molecular basis for mammalian echolocation has been receiving much concerns. Recent findings on the parallel evolution of *prestin* sequences among echolocating bats and toothed whales suggest that adaptations for high-frequency hearing have occurred during the evolution of echolocation. Here, we report that although the species tree for echolocating bats emitting echolocation calls with frequency modulated (FM) sweeps is paraphyletic, *prestin* exhibits similar functional changes between FM bats. Site-directed mutagenesis shows that the amino acid 308S in FM bats is responsible for the similar functional changes of *prestin*. We strongly support that the occurrence of serine at position 308 is a case of hemiplasy, caused by incomplete lineage sorting of an ancestral polymorphism. Our study not only reveals sophisticated molecular basis of echolocation in bats, but also calls for caution in the inference of molecular convergence in species experiencing rapid radiation.

Key words: **echolocation, homoplasy, hemiplasy, SLC26A5, bat.**

Letter

Echolocation is a specific sensory system, the efficacy of which critically depends on both emission and subsequent hearing of high frequency calls. The integration of fossil and molecular phylogenetic evidence suggests that laryngeal echolocation might have originated in the last common ancestor of bats (Springer et al. 2001) Subsequent evolution led to numerous modifications in call features, which permitted echolocating bats to adapt their hunting behaviors to specific acoustic environments (Jones and Holderied 2007). Based on call frequency structures, the echolocation calls of bats are generally categorized into two major types: frequency modulated (FM) sweeps and constant frequency (CF) tones (Schnitzler et al. 2003). Interestingly, FM echolocating bats are not monophyletic in the species tree (Teeling et al. 2005), which provids a valuable system for exploring the molecular basis of improvement in FM echolocation because when echolocation originated, it was too rudimentary to allow bats to estimate distance accurately (Boonman et al. 2014).

A number of studies have examined the molecular basis of mammalian echolocation. Some hearing genes have been identified as candidates due to their parallel evolution at sequence level among echolocating mammals (Li et al. 2010; Liu et al. 2010; Davies et al. 2011; Liu et al. 2011; Shen et al. 2012; Parker et al. 2013). One of the most interesting candidates is the hearing gene *prestin*. A statistical analysis showed that the number of observed parallel amino acid replacements of prestin in echolocating mammals significantly exceeds that expected due to chance (Li et al. 2010), suggesting that shared selection, rather than chance, underlies the observed parallel substitutions. Moreover, *prestin* is functionally convergent among echolocating mammals, and the parallel amino acids that have been identified account for the functional convergence (Liu et al. 2014). This provides strong evidence that selection has acted to promote the parallel substitutions of *prestin* genes in echolocating mammals.

To unravel the relationships between *prestin* and the echolocation in FM bats, we first tested for the presence of differential selection on *prestin* in FM and CF bats by estimating the ratio (ω) of non-synonymous to synonymous substitution rates using a likelihood method (Yang 2007). Assuming that the *prestin* sequences from all branches in the tree of the 31 taxa (supplementary fig. S1, Supplementary Material online) have the same ω (model A in table 1), we estimated that $\omega = 0.0877$. If each branch was assumed to have its own ω (Model B in table 1), we found that Model A was significantly different from Model B ($P < 0.0001$), suggesting that the evolutionary forces acting on *prestin* genes varied among different bat lineages. Next, we tested whether a model that allows different ω values for FM and CF bats (model C in table 1) fits the data significantly better than a simpler model that does not allow the difference (model D in table 1). Indeed, model C fits the data significantly better than model D ($P = 0.033$; table 1), suggesting that selection for *prestin* in FM bats was significantly different from that in CF bats. Thus, the function of *prestin* in FM and CF bats might have diverged and adapted to their specific acoustic environments and echolocation calls.

Prestin encodes an anion transporter, driving the electromotility of mammalian outer hair cells in the hearing organ of Corti (Zheng et al. 2000). The voltage-dependent nonlinear capacitance (NLC) is often used to evaluate the function of

Table 1. Likelihood Ratio Tests of Selective Pressures on *Prestin* in Bats.

Models	ω (d_N/d_S)	ln*L*[a]	np[b]	Models Compared	*P* values
A. All branches have one ω	ω = 0.0877	−13874 8	55		
B. Each branch has its own ω	variable ω by branch	−13752 1	107	A vs. B	<0.0001
C. Ancestral branches of FM bats have ω_1, Ancestral branches of CF bats have ω_2	$\omega_1 = 0.284$, $\omega_2 = 0.737$	−13840 2	57		
D. Ancestral branches of FM and CF bats have the same ω ($\omega_1 = \omega_2$)	$\omega_1 = \omega_2 = 0.4915$	−13842 4	56	C vs. D	0.033

[a]Natural logarithm of the likelihood value.
[b]Number of parameters.

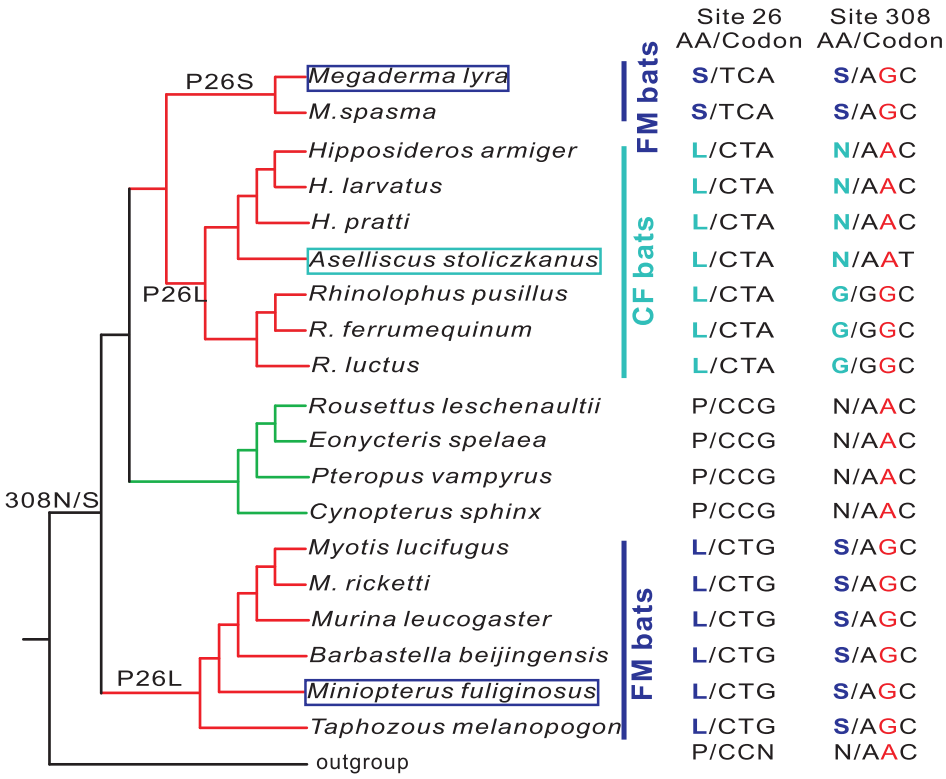

Fig. 1. Phylogenetic relationships of bats with intact *prestin* sequences. Red branches correspond to echolocating bats. Green branches indicate non-echolocating bats. The boxed in names show the species that were selected for experimental analyses of their *prestin* genes. The blue and cyan bars correspond to FM and CF bats, respectively. Amino acids and codons of the sites 26 and 308 are shown, and the polymorphic sites at the second position of codon 308 are highlighted with red color.

prestin, which is qualified using three parameters $1/\alpha$, $V_{1/2}$, and Q_{max}/C_{lin} by fitting a two-state Boltzmann function (Santos-Sacchi 1991; Dallos and Fakler 2002). We characterized the function of *prestin* in FM bats by examining the greater false vampire bat *Megaderma lyra* and the eastern bent-wing bat *Miniopterus fuliginosus* from the two suborders of Yinpterochiroptera and Yangochiroptera respectively. In comparison to a CF bat, Stoliczka's trident bat (*Aselliscus stoliczkanus*), the function of *prestin* was significantly different in FM bats, which is consistent with the evolutionary analyses. Interestingly, the function of *prestin* in *Miniopterus fuliginosus* was similar to that of *Megaderma lyra*, which is more closely related to *Aselliscus stoliczkanus* in the species tree (fig. 1). Although the two parameters $1/\alpha$ and Q_{max}/C_{lin} were not significantly different, the functional parameter $V_{1/2}$ of prestin in CF bats significantly shifted in the direction of depolarization in comparison with that of FM bats ($P < 0.001$, Student's *t*-test; fig. 2*A*), indicating that there are functional differences in *prestin* between FM and CF bats. $V_{1/2}$ is the voltage at peak capacitance or half-maximal nonlinear charge transfer. The depolarizing shift in the voltage-dependent nonlinear peak capacitance in CF bats can increase the chloride allosteric binding site affinity for chloride transitions across the membrane with a consequence of increasing NLC, possibly resulting in higher frequency hearing (Ashmore 2008; Oliver et al. 2001). In addition, our previous study showed a significant correlation between the $V_{1/2}$ values and best hearing frequencies in mammals (Liu et al. 2014). Interestingly, the depolarizing shift of the $V_{1/2}$ values in CF bats is consistent with the observation that CF bats can generally detect higher

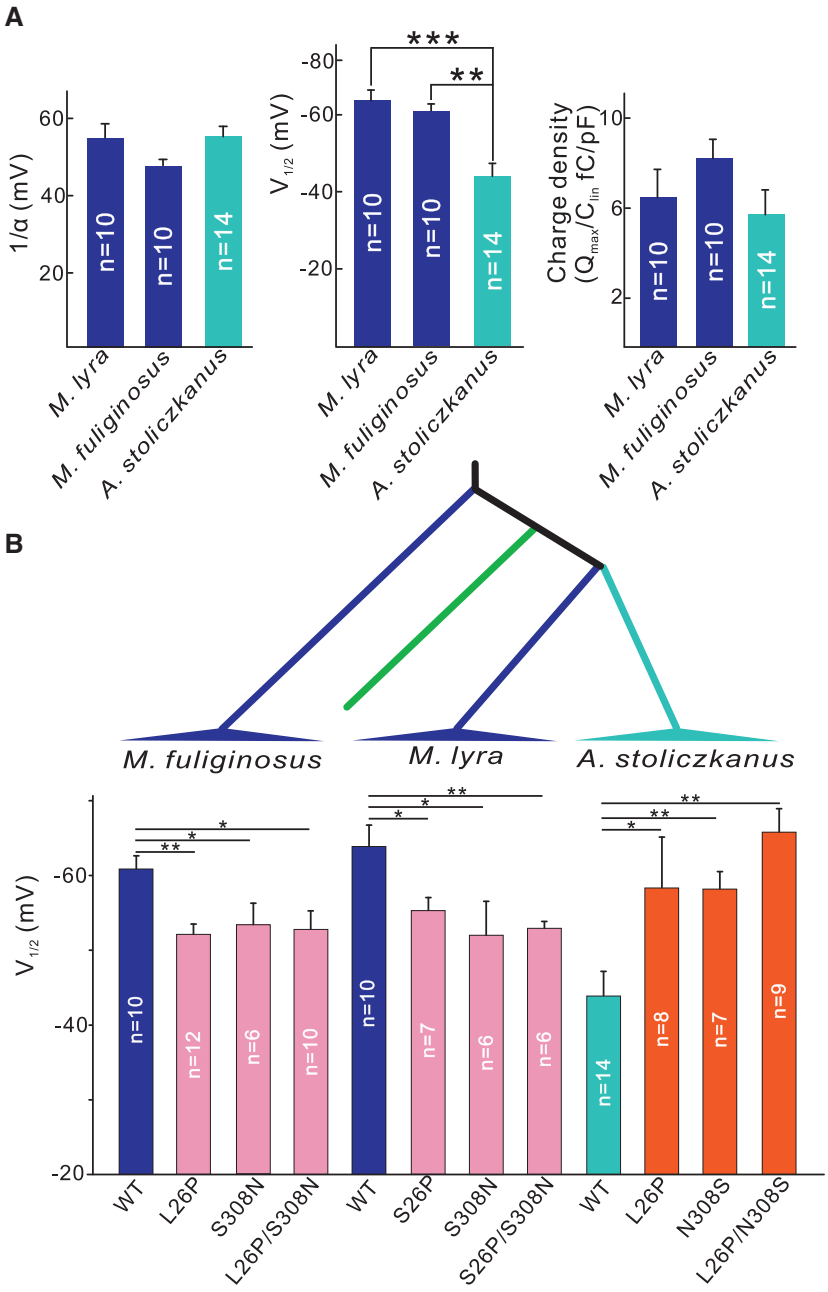

Fig. 2. Functional tests of *prestin* in FM and CF bats. (*A*) Three functional parameters 1/α, $V_{1/2}$, and Q_{max}/C_{lin} were obtained by fitting curves of non-linear capacitance with the two-state Boltzmann function. These parameters were compared among three bat species: *Megaderma lyra*, *Miniopterus fuliginosus*, and *A. stoliczkanus*. FM and CF bats are indicated by blue and cyan colors, respectively. (*B*) Simplified species tree of bats. The green lineage indicates the non-echolocating bats. The site 308S and the replacement P26L/S are responsible for the similar functional changes of $V_{1/2}$ of *prestin* between FM bats. Different colors show the different mutants for the 26 and 308 amino acids in the FM and CF bats. All values are presented as the mean ± S.E. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

frequency sound (in general >100 kHz) than those in FM bats (in general 50–70 kHz) (Schnitzler et al. 2003).

To identify the potential amino acids underlying the molecular mechanisms for the similar functional changes in FM bats, we separately inferred the sequence of *prestin* on the ancestral branches leading to two different clades of FM bats based on the species tree using maximum-likelihood and maximum-parsimony methods of PAML4.0 (Yang 2007). In comparison with the *prestin* sequences from the extant species according to the methods described in previous studies (Li et al. 2010; Liu et al. 2012, 2014), we identified a replacement N308S that appeared on both of the ancestral branches of FM bats (fig. 1). To determine whether this replacement represented a transition from Asn to Ser at position 308 that occurred independently on the ancestral branches of FM bats (homoplasy), or it was a polymorphic site where Asn and Ser co-existed in the prestin of bat ancestors but only Ser became fixed later in the ancestors of FM bats (hemiplasy) (Hahn and Nakhleh 2016; Storz 2016), we examined the codon composition of this amino acid in all bats. The codons encoding Asn

in CF bats and Ser in FM bats primarily differed in the second position. In FM bats, the second position of codon at site 308 is G, but in the CF bats from *Hipposideros*, the second position is A, which is consistent with the pattern in non-echolocating bats and the outgroup (fig. 1). Notably, the second position is also G in the CF bats from *Rhinolophus*. If the transition from A to G was a parallel substitution, it should have occurred independently three times in the most recent common ancestors (MRCAs) of megadermatid, yangochiropteran, and rhinolophoid bats. However, multiple independent origins for the same substitution most unlikely occurred during a relatively short period when bats had proceeded rapid radiation. The only remaining reasonable explanation is the incomplete lineage sorting of site 308 in the *prestin* sequences of bats; namely, a polymorphic nucleotide A/G at the second position of codon 308 appeared in the MRCAs of bats and later became fixed independently in the MRCAs of megadermatid, yangochiropteran, and rhinolophoid bats.

To determine whether the amino acids 308S was responsible for the similar functional changes of *prestin* in FM bats, we first created mutants of this site based on the genetic backgrounds of FM bats: *M. fuliginosus* and *M. lyra*, respectively. By comparing these bats with their corresponding wild types, the values of $V_{1/2}$ from the single site mutants shifted significantly in the direction of depolarization (fig. 2*B*; $P < 0.05$, Student's *t*-test) as seen in CF bats. This shift indicates that Ser is fixed at site 308 and provides evidence for the similar functional changes of *prestin* in FM bats. To confirm these results, we replaced Asn with Ser at site 308 in *prestin* of the CF bat *A. stoliczkanus* and found that the values of $V_{1/2}$ of the mutant shifted significantly in the direction of hyperpolarization (fig. 2*B*; $P < 0.01$; Student's *t*-test). The mutation and rescue experiments clearly indicate the important implications of the site 308S for the functional effects of *prestin* in FM bats, and provide novel insights into our understanding of the molecular mechanisms of echolocation.

In addition, we observed that the 26th substitution of prestin was leucine on the ancestral branch leading to the yangochiropteran FM bats and that the same replacement P26L occurred on the ancestral branch of CF bats due to the shared transition from C to T at the second position of the codon at site 26 (fig. 1). A statistical test (Zhang and Kumar 1997) showed that one parallel site was significantly greater than random expectations ($P < 0.05$), suggesting that selection, rather than chance, underlies the observed parallel substitution. Thus, the parallel replacement of P26L between FM and CF bats appears to be closely linked to echolocation. Interestingly, this site was changed into serine on the ancestral branch that led to the megadermatid FM bats (fig. 1). Moreover, the mutation and rescue experiments showed that this divergent site was also responsible for the similar functional changes of *prestin* in FM bats (fig. 2*B*). We next created the double replacement mutants L/S26P and S308N for the two FM bats *M. fuliginosus* and *M. lyra*. As expected, the values of $V_{1/2}$ from the double mutants significantly shifted in the direction of depolarization in comparison with their corresponding wild types. However, to our surprise, the change in the magnitude of prestin function in the double mutants was similar to those observed in the single-site mutants. It is worth noting that there may be multiple sites responsible for functional changes of genes, but that the combination of these sites may not influence gene function by additive effects.

Why is the divergent site P26L/S observed, although the site 308S can alone explain the similar functional changes of *prestin* in FM bats? One likely scenario is that the site 308S accounted for the shifting in the direction of hyperpolarization of the prestin protein in FM bats; however, this replacement also influenced other aspects of prestin function, which required other amino acid substitutions to compensate for the additional functional defects. Indeed, when only the amino acid replacement S308N was created in the prestin of *M. fuliginosus*, the functional parameters Q_{max}/C_{lin} and $1/\alpha$ increased significantly compared to the wild type ($P < 0.01$, Student's *t*-test; supplementary fig. S2, Supplementary Material online). Similarly, the value of Q_{max}/C_{lin} became larger in the single mutant S308N of *M. lyra* than in its wild type. To further verify the robustness of these results, we examined the functional effects of the single mutant N308S of prestin in the CF bat *A. stoliczkanus*. We found that the functional parameters Q_{max}/C_{lin} and $1/\alpha$ in the single mutant prestin decreased significantly ($P < 0.05$, Student's *t*-test). These results demonstrated that the site 308S not only generated the similar functional effects at the parameter $V_{1/2}$ between the FM bats, but also led to other functional changes of prestin that may not aid the ability of FM bats to hear their particular echolocation calls.

Numerous studies have shown that parallel and divergent amino acid substitutions underlie the similar functional changes of genes (Zhang 2003; Yokoyama et al. 2008; Rosenblum et al. 2010; Natarajan et al. 2015). For *prestin*, we observed that an apparent parallel substitution N308S was associated with similar functional changes in FM echolocating bats. However, the substitution actually only occurred once in the MRCA of bats and the ancestral polymorphism was retained in FM bats. These results provide novel insights into our understanding of the evolutionary and molecular mechanisms of echolocation in bats, also calls for the caution in the inference of molecular convergence, especially in those species undergoing the rapid radiation.

Materials and Methods

To estimate selection on echolocating bats emitting FM and CF echolocation calls, we downloaded the coding sequences of *prestin* genes of 31 taxa from GenBank (supplementary table S1, Supplementary Material online). These sequences were initially aligned using MUSCLE (Edgar 2004) followed by manual adjustments (supplementary fig. S3, Supplementary Material online). Maximum-likelihood method was used to estimate the selective pressures using branch models in codeml from the PAML package (Yang 2007) based on the mammalian species tree. The ratio of non-synonymous to synonymous substitution rates, termed ω, was used to estimate the mean selection pressures on different branches of the tree. First, we estimated ω across the tree under a one-

ratio model. Next, we estimated an independent ω value for each branch under the free-ratio model. Finally, we used the two-ratio branch model to compare the estimated ω values on specific foreground branches in the phylogeny with the background values. We chose two distantly related FM echolocating bats (*Megaderma lyra* and *Miniopterus fuliginosus*) and one CF echolocating bat Stoliczka's trident bat (*A. stoliczkanus*) to conduct experimental analyses. We amplified the intact coding sequences of prestin and cloned into these expression vector pEGFP-N1 (Clontech), yielding C-terminal GFP fusion constructs. Site mutations were introduced into the wild-type prestin vectors using a PCR-based site-directed mutagenesis. HEK293 cells were transiently transfected with the wild-type or mutant *prestin*-GFP vectors. Positive cells with strong and luminous membrane fluorescence were selected for electrophysiological experiments to record the nonlinear capacitance (NLC) by whole-cell patch-clamp recording using the EPC 10 USB amplifier (HEKA Instruments Inc.), as described in Liu et al. (2014). NLC was quantified by fitting the derivative of a two-state Boltzmann function:

$$C_m = \frac{Q_{max}\alpha}{\exp[\alpha(V_m - V_{1/2})](1 + \exp[-\alpha(V_m - V_{1/2})])^2} + C_{lin}$$

The parameters $1/\alpha$, $V_{1/2}$ and Q_{max}/C_{lin} were extracted to quantify the function of *prestin*. Q_{max} is the maximum charge transfer, $V_{1/2}$ is the voltage at which the maximum charge is equally distributed across the membrane, C_{lin} is the linear capacitance and α is the slope factor of the voltage dependence of charge transfer.

Supplementary Material

Supplementary table S1 and figures S1–S3 are available at *Molecular Biology and Evolution* online.

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