Adaptive evolution of hearing genes highlights multiple evolutionary paths for dolphin echolocation in different environments

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Abstract

Echolocation was a key development in toothed whale evolution, favoring their ecological success and adaptive radiation across multiple habitats. Previous bioacoustic and morphological studies suggest that environmental pressures have influenced the evolution of echolocation in odontocetes. This hypothesis demands further investigation, especially regarding molecular mechanisms involved in odontocetes' adaptive radiation across multiple habitats. Here we show that the coding sequences of four hearing genes involved in echolocation (CDH23, SLC26A5, TMC1 and CLDN14) have different signatures of molecular evolution among riverine, coastal and oceanic dolphins, suggesting that the evolutionary constraints of these habitats have shaped the genetic diversity underlying toothed whale sonars. Our comparative analysis across 37 odontocete species has revealed patterns of accelerated evolution within coastal and riverine lineages, supporting the hypothesis that these habitats generate specific selective pressures to sonar propagation, which are not found in the ocean (e.g., clutter and reverberation). Specific branches with sites under diversifying selection include: three coastal/estuarine species which have recently diverged from closely-related lineages inhabiting freshwater (*Cephalorhynchus commersonii*, *Sotalia guianensi*s and *Orcaella heinsohni* - CDH23), and three species that operate specialized Narrow Band High Frequency Sonars (*Phocoena sinus* - SLC26A5, *Neophocaena phocaenoides* and *Cephalorhynchus commersonii* - CDH23). All positively selected lineages are coastal or estuarine from shallow waters (< 100), corroborating current knowledge on these species' sonars. Among river dolphin branches, positive selection was found on specific sites of the genes CDH23 and SLC26A5, when compared to other cetaceans and Laurasiatherians. Finally, the detection of sites with different overall substitution rates (dN/dS) among river and marine dolphins, together with the finding of different amounts of positively selected sites in each environmental group, suggests distinct selective pressures acting in these groups in the molecular evolution of echolocation.

Keywords: cetaceans, Odontoceti, molecular evolution, natural selection, divergence, convergence

Background

Echolocation was a key development in toothed whale evolution, favoring their ecological success and adaptive radiation across multiple habitats: from shallow, murky rivers in South America to deep oceans in East Asia (Berta, 2015). In underwater environments, where vision is often limited, this sensory system allows dolphins to navigate, and scan their surroundings by producing and receiving ultra-frequency sounds (Au, 1993).

The remarkable diversity of odontocete species is reflected in their morphological and behavioral characteristics, as well as the various habitats they occupy, which encompass a gradient of physical conditions such as depth, density, salinity, and pressure. These conditions affect the propagation of sound underwater and thus influence the operation of dolphin sonars, creating different selective pressures in each environment(Ketten & Wartzok, 1990). For example, in shallow and spatially complex habitats such as rivers, estuaries and coastal waters, sound waves encounter more obstacles and generate unwanted echoes, limiting sound propagation. Such constraints have likely favored the evolution of short-range sonars in river and coastal dolphins, where several features (e.g. high frequency, narrow-band, lower source level) allow prey detection in places with high levels of reverberation and clutter (Jensen et al., 2013; Kyhn et al., 2009; Ladegaard et al., 2015). Oceanic dolphins, on the other hand, inhabit more stable and open spaces (i.e. ocean waters), with less constraints to sound propagation and thus a broader search range. Most off-shore and pelagic dolphins employ lower ultrasonic frequencies with longer wavelengths that facilitate target detection over greater distances (Ketten & Wartzok, 1990). This evidence suggests that environmental conditions have played a role in shaping the acoustic parameters of the biosonar in toothed whales. However, what evolutionary patterns emerged from this diversification across distinct habitats? Regarding the behavioral and acoustic features, it has been largely recognized that odontocetes are clustered into acoustic groups of higher and lower frequency echolocators, although the number and composition of these categories remains unsettled (Au 200..;). Moreover, dolphins are able to perform subtle adjustments in their biosonars (e.g. frequency)

in face of environmental changes such as moving to a deeper or more clustered area while foraging.

The evolution of echolocation in different environments appears to have shaped convergent cochlear morphologies among riverine, coastal and marine dolphins. For instance, different eco-morphologies of the periotic (i.e. shape of the cochlear duct and pars cochlearis) correspond with frequency range and narrow-band vs. bimodal echolocation, reflecting environmental preferences of extant and extinct dolphins (Gutstein et al., 2014). Furthermore, cochlear morphology not only recovers phylogenetic relationships among odontocetes, but also reflects habitat preferences of freshwater, coastal and marine species, through well-marked differences in overall shape and coiling, number of turns and location of the fenestra vestibulii of the cochlea between these groups.

Echolocation relies on specialized high frequency hearing, whose early emergence in toothed whale ancestors was fundamental to the sonars of modern species (Ketten, 1992; Liu et al., 2018; Park et al., 2016). Among mammals, high frequency hearing is largely mediated by the inner and outer hair cells of the auditory cochlea. Hair cell function and development are regulated by several known genes [...]. In outer hair cells, hearing adjustment to higher frequencies is mediated by the motor protein *Prestin*. In inner hair cells, the transmembrane channel-like protein TMC1 is required for hair cells maturation and survival, as well as stereocilia movement (Pan et al., 2018). The conversion of mechanical stimuli into electrochemical signaling is regulated by a complex of cadherins. The gene CDH23 encodes a caderin in this complex that binds the extremities of hair bundles and is required for both their movement and development, contributing to regulate the ion flow in and out the hair cells (Kazmierczak et al., 2007). The ionic gradient of hair cells is also maintained by the adhesion protein claudin-14, encoded by CLDN14. Increasing evidence suggests that hearing genes are involved in functionally relevant pathways in echolocation and were targeted by natural selection in several dolphin lineages that evolved in different environments. However, genome-wide studies indicate that there is a large pool of unexplored hearing genes correlated with cetaceans' adaptation to different habitats (Chikina et al., 2016; Foote et al., 2015).

A growing body of evidence shows the importance of hearing genes in cetacean evolution, as they have experienced multiple events of positive selection in odontocete lineages, as well as convergent evolution among echolocating mammals - i.e. toothed whales and bats (**CDH23:** (Lambert et al., 2017); **CLDN14:** Xu et al. (2013); **TMC1:** (Davies et al., 2012); **SLC26A5:** (Liu et al., 2014, 2018; Y. Li et al., 2010). Taken together, this evidence suggests that several hearing genes with functional relevance to echolocation were targeted by natural selection in dolphin lineages that evolved in different environments.

Here we selected four candidate hearing genes (CDH23, CLDN14, SLC26A5, TMC1) to address the question of how echolocation has diversified among toothed whales in different habitats. Using multiple evolutionary models, we performed a comparative analysis of these genes across all odontocete families to investigate patterns of molecular evolution among riverine, coastal and oceanic lineages. We hypothesize that the different evolutionary pressures underlying the evolution of echolocation in each environment resulted in different patterns of molecular evolution, regarding positive selection and adaptive convergence, within hearing genes.

Results

Our selection analyses recovered differences in the molecular evolution of hearing genes between riverine, coastal and oceanic dolphins, highlighting stronger selective pressures among coastal lineages.

Branch-models

We detected gene-wide signals of diversifying episodic selection affecting at least one site on at least one branch among all odontocetes for CDH23 and TMC1. Comparing groups of dolphins from different habitats (riverine, coastal and oceanic), only coastal dolphins showed signatures of positive selection, for the genes CDH23, TMC1 and SLC26A5. Similarly, signatures of selection intensification within coastal odontocetes (CDH23, TMC1) were found by RELAX, suggesting that hearing genes have experienced events of accelerated evolution among dolphins inhabiting coastal and estuarine environments, when compared to other cetaceans (Odontoceti + Mysticeti) and artiodactyls (*Sus scrofa* + *Bos taurus*). Selection relaxation was also detected on CDH23 among all odontocetes, and for riverine and oceanic

dolphins, which is consistent with the absence of positive selection signatures for these groups on BUSTED.

Table 1. Hypotheses investigated to unravel the evolutionary history of echolocation in odontocetes

Hypothesis	Foreground/test branches
1a	Odontoceti ancestor
1b	Extant Odontoceti
1c	All Odontoceti branches (extant + ancestors)
2a	Riverine Odontoceti (*S. fluviatilis, N. a. asiaeorientalis, L. vexillifer, I. geoffrensis, P. gangetica, O. brevirostris, P. blainvillei*)
2b	Phenotypically riverine Odontoceti (*L. vexillifer, I. geoffrensis, P. gangetica, P. blainvillei*)
2c	Odontoceti that inhabit rivers (*S. fluviatilis, N. a. asiaeorientalis, L. vexillifer, I. geoffrensis, P. gangetica, O. brevirostris*)
3	Oceanic Odontoceti
4	Coastal Odontoceti
5	Riverine + Marine (Oceanic + Coastal) Odontoceti

Table 2. RELAX results for the genes with evidence of shifts (intensification or relaxation) in the strength of natural selection. K = selection intensity parameter. LR = likelihood ratio test.

RELAX - branch-wide strength of natural selection					
Gene	**Test branches**	**Selection shift**	**K**	**p-value**	**LR**
CDH23	Extant odontocetes	relaxation	0.61	0.009	6.90
	Riverine odontocetes	relaxation	0.69	0.000	22.04
	Oceanic odontocetes	relaxation	0.67	0.003	9.12
	Coastal odontocetes	intensification	1.57	0.000	14.32
TMC1	Coastal odontocetes	intensification	3.64	0	15.15
CLDN14	Extant odontocetes	intensification	1.53	9	6.74

Branch-site models

We found a total of 5 lineages experiencing episodic diversifying selection, all of them coastal odontocetes (Table 3). CDH23 showed the highest number of lineages under positive selection (3 - *Cephalorhynchus commersonii*, *Sotalia guianensis* and *Orcaella heinsohni*), while one positively selected lineage each was recovered for SLC26A5 (*Phocoena sinus*) and TMC1 (*Neophocaena phocaenoides*). No evidence was found for episodic diversifying selection among the riverine or oceanic lineages, suggesting that the positive selection events among these lineages were weaker or fewer compared to coastal dolphins.

The results for codeml's branch-site test of positive selection mostly corroborate what we found with BUSTED and aBSREL, with evidence of positive selection among coastal dolphins for CDH23, SLC26A5 and TMC1 (Table 4). suggesting that several sites among river dolphin lineages have experienced positive selection for CDH23.

Table 3. aBSREL results for the genes with evidence of episodic diversifying selection affecting some branches in the tree. B = Optimized branch length. LRT = Likelihood ratio test statistic for selection. Test p-value = p-value corrected for multiple testing.

aBSREL - branches under episodic diversifying selection						
Gene	**Species**	**B**	**LRT**	**Test p-value**	**Uncorrected p-value**	**ω distribution over sites**
CDH23	*Cephalorhynchus commersonii*	0.0000	11.3691	0.0152	0.0012	ω1 = 0.0630 (100%) ω2 = 1120 (0.25%)
	Sotalia guianensis	0.0000	11.2033	0.0152	0.0013	ω1 = 0.00 (100%) ω2 = 1350 (0.10%)
	Orcaella heinsohni	0.0000	10.2908	0.0222	0.0020	ω1 = 0.00 (100%) ω2 = 1340 (0.33%)
SLC26A5	*Phocoena sinus*	0.0000	10.8594	*0.0197*	0.0015	ω1 = 0.00 (100%) ω2 = 558 (0.31%)
TMC1	*Neophocaena*	0	329709	0	0	ω1 = 0.00 (100%)

phocaenoides		ω2 = 100000 (0.31%)

Table 4. Codeml results for the genes with evidence of positive selection among individual lineages and sites. lnL = log likelihood of the corresponding model. LRT = 2 *[(lnL of the alternative model "Amodel" - lnL of the null model)]. np = number of parameters. df = degrees of frequency. p value corresponds to the statistical support obtained by chi-square assessments.

Gene	codeml model	lnl	LRT	np	df	p value	Positively Selected Sites (BEB > 0.9)*
CDH23	Amodel (H2)	-27045.18	3.95826	90	1	0.0466	60 M 0.941; 2288 N **0.953***; 2755 - 0.923; 3312 K 0.926
	Nullmodel (H2)	-27047.17		89			
	Amodel (H3)	-27,053.81	8.646272	90	1	0.0032	625 V 0.843
	Nullmodel (H3)	-27,058.13		89			
	Amodel (H4)	-27,040.89	5.307914	90	1	0.0212	1429 D 0.909; 1911 T 0.881; 3269 Q 0.932
	Nullmodel (H4)	-27,043.54		89			
SLC26A5	Amodel (H4)	-5,722.90	4.603326	90	1	0.0319	296 A 0.870
	Nullmodel (H4)	-5,725.20		89			
TMC1	Amodel (H1b)	-5,189.40	8.280894	90	1	0.004	163 N 0.942; 274 L 0.907; 330 - **0.955***; 344 - **0.956***; 669 S 0.944
	Nullmodel (H1b)	-5,193.54		89			
	Amodel (H4)	-5,187.13	14.306606	90	1	0.0001	330 - **0.987***; 344 - **0.987***
	Nullmodel (H4)	-5,194.29		89			

Site models

Contrast-FEL recovered sites with significantly different ω ratios among riverine and marine dolphins, suggesting that some sites in the genes CDH23, TMC1 and SLC26A5 have evolved under different selective pressures and natural selection rates between odontocetes from marine and freshwater environments (Table 4).

Table 5. Contrast-FEL results for differential selection among river and marine dolphins. The column R x M depicts the sites that showed significantly different ω ratios among these two groups. ω riverine, ω marine and ω background represent the ω values for these sites for each dolphin group and for the background lineages, respectively.

Gene	Total sites	Sites with different dN/dS ratios				p-value
		R x M	ω riverine	ω marine	ω background	
CDH23	3365	15	0.176	0.168	0.104	0.1
SLC26A5	747	2	0.132	0.144	0.157	0.1
TMC1	766	3	0.382	0.124	0.125	0.1
CLDN14	243	1	0.0273	0.0156	0.0919	0.1

Discussion

Echolocation and high frequency hearing triggered odontocetes adaptive radiation through a diversity of habitats, exposing their phenotypic and genotypic characters to the different selective pressures in each environment. Here we show that the coding sequences of four hearing genes involved in echolocation (CDH23, SLC26A5, TMC1 and CLDN14) present different signatures of molecular evolution among riverine, coastal and oceanic species, suggesting that the different evolutionary constraints of these habitats have shaped the genetic diversity underlying toothed whales' sonars. Our comparative analysis across 37 odontocete species has revealed patterns of accelerated evolution within coastal and riverine lineages, supporting the hypothesis that these habitats generate specific selective pressures to sonar propagation, which are not found in the ocean.

Our findings of positive selection and selection intensification occurring exclusively within coastal lineages suggest that the coastal environment provided the strongest selective pressures to the evolution of hearing genes involved in echolocation. The five lineages experiencing episodic diversifying selection are known to inhabit shallow waters (< 100 m deep) and are found in both coastal and estuarine environments. Shallow habitats with low visibility and high obstacle density, such as estuaries and rivers, are more acoustically complex than oceanic waters, and prey detection in these environments is usually limited by

clutter (i.e. unwanted echoes from surrounding objects), creating specific pressures that selected for short range, high frequency sonars in multiple odontocete species (Jensen et al., 2013; Ladegaard et al., 2015).

Additionally, three of the five lineages under positive selection have recently diverged from their riverine sister species (*S.guianensis* and *S.fluviatilis* - X mya; *O.heinsohni* and *O.brevirostris* - X mya; *N.phocaenoides* and *N.asiaeorientalis* asiaeorientalis - X mya) (McGowen et al., 2020). Taken together, our results show increased rates of evolution and positive selection in hearing genes among coastal species, which could be correlated to their recent diversification in clutter limited habitats.

Surprisingly, no lineage-enriched signatures of positive selection were recovered for river dolphins. However, the combined evidence for positive selection affecting multiple sites in CDH23 and selective relaxation across river dolphin branches suggests that these lineages underwent convergent shifts in their evolutionary regimes. These results might indicate that CDH23 has experienced an accelerated evolution caused by relaxation of selective constraints, which could be associated with both non-adaptive and adaptive processes. More importantly, we uncovered signatures of molecular convergence among river dolphins, which are known to share extensive morphological convergence associated to their independent colonization of freshwater habitats (Hamilton et al. 2001). Furthermore, high frequency, low source and short range sonars have been reported in four species of river dolphins and hypothesized to be adaptive under the selective constraints of riverine environments (*Neophocaena asiaeorientialis asiaeorientialis* - (S. Li et al., 2005); *Platanista gangetica* and *Orcaella brevirostris* - (Jensen et al., 2013); Inia geoffrensis - (Ladegaard et al., 2015).

Among the four genes we investigated, CDH23 showed the highest numbers of lineages under positive selection and sites with different evolutionary rates among riverine and marine dolphins. CDH23, SLC26A5 and TMC1 have been previously reported under positive selection in dolphin lineages, however, most past studies have analyzed only one or two dolphin species and were focused on cetacean adaptation during their transition from terrestrial to aquatic habitats. Our study is the first to our knowledge to show an underlying molecular basis to the divergence of echolocation during odontocetes' adaptive radiation.

The patterns of accelerated evolution we report here might also be correlated to other evolutionary scenarios not determined by environmental differences. For example, three of the coastal lineages under diversifying selection (N. phocaenoides, P. sinus, C. commersonii) are Narrow Band High Frequency (NBHF) echolocators, meaning that they operate sonars within a 125-140 kHz peak frequency range with narrow bandwidth (11-20 kHz). NBHF sonars are thought to have evolved independently in four extant odontocete families (Pontoporiidae, Kogiidae, Phocoenidae and Delphinidae) under the selective pressures of predation by killer whales and other extinct raptorial odontocetes (Galatius et al., 2018). Therefore, our results could be reflecting a case of molecular convergence among NBHF species. Conversely, our findings could also be influenced by pleiotropic effects of the studied genes.

Conclusions

Methods

Sequence download and alignment

We selected five candidate hearing genes: CDH23, SLC26A5, TMC1, CLDN14 and SMPX, and recovered their coding sequences from the Nucleotide, Sequence Read Archive (SRA) and Genome databases in Genbank. We included 42 species of cetaceans (37 odontocetes and 5 baleen whales - Supplementary Table 1), along with two external Artiodactyla lineages: *Sus scrofa* and *Bos taurus*. Isolated gene sequences were downloaded in fasta format from the Nucleotide database. SRA sequences were obtained from a concatenated alignment of 3191 coding genes (McGowen et al., 2020), which was then aligned against the sequences of the candidate genes for *Orcinus orca* and *Tursiops truncatus* to recover cetacean orthologs, using MAFFT version 7 online (Katoh et al., 2019). The genomic sequences of *Platanista gangetica* and *Phocoena sinus* were downloaded from the Genome database and coding sequences for the candidate genes were extracted using BLAST version 2.12.0 (Johnson et al., 2008), since isolated genes or SRA sequences were not available for these species. Additionally, we assembled the coding sequences of *Sotalia guianensis* and *Sotalia fluviatilis* from newly sequenced whole genomes, using BLAST to locate the sequences, a python script (Supplementary material) for extraction, and Aliview

version 1.27 (Larsson, 2014) for alignment, assembly and visual inspection of the gene fragments.

Phenotypic and environmental categories

To compare evolutionary patterns in hearing genes among toothed whales from different habitats, we first assigned each species to an environmental category among "riverine", "coastal", "marine", and "polar", according to their habitat preferences (Berta, 2015; Webber et al., 2015; Würsig et al., 2017). When the species showed no predominant habitat, they were assigned to a fifth category termed "generalist", to avoid confounding effects in the association between molecular changes and different environments.

To assess the diversity of acoustic properties in odontocetes' sonars, we used the classification proposed by (Jensen et al., 2018) and assigned each odontocete species to one of the four sonar categories described in the study.

Sequence alignment

To perform selection and convergence analyses, we assembled the gene and protein sequences into multi-species alignments with MAFFT online, applying the G-INS-i alignment strategy (remaining settings were kept on default) (Katoh et al., 2019). To access protein sequences, we translated the alignments into amino acids using Aliview (Larsson, 2014). Finally, we used PAL2NAL (Suyama et al., 2006) to obtain the codon alignments used in all selection analyses described below. All multi-species codon alignments were visually inspected for quality screening to remove stop codons, gaps and insertions, correct misaligned sequences/nucleotides, and exclude sequences responsible for frame-interruption. A total of 10 multi-species alignments were generated, each containing 44 species (Supplementary table 2).

Gene tree reconstruction

We used W-IQ-TREE multicore version 1.6.12 (Trifonopoulos et al., 2016) to reconstruct maximum likelihood gene trees for each multi-species codon alignment. In order to evaluate branch support of the consensus trees, Ultrafast Bootstrap Analysis was performed with 10000 bootstrap replicates, 1000 maximum iterations and a minimum

correlation coefficient of 0.99. The following single branch tests were conducted to assess the support for Maximum Likelihood Trees: SH-aLRT branch test was set for 5000 replicates and Approximate Bayes test was selected to maximize the confidence of the tree. Default settings were maintained for the remaining available parameters and the best-fit model based on ModelFinder analysis was GY+F+G4.

Selection analyses

To investigate the behavior of natural selection on the coding sequences of the five hearing genes, we performed selection analyses to detect positive selection, nucleotide substitution rates and selective constraints on branches and sites using a combination of codon models implemented on the HyPhy (Kosakovsky Pond, Poon, et al., 2020) and PAML (Yang, 2007) program packages. On Hyphy, we used BUSTED and RELAX for branch-level analyses, aBSREL for branch-site analyses, and MEME, FUBAR and Contrast-FEL for site-level analyses (Kosakovsky Pond, Wisotsky, et al., 2020; Murrell et al., 2012, 2013, 2015; Smith et al., 2015; Wertheim et al., 2015). On PAML, we estimated the codeml models 0 (one-model), 1 (free-model) and 2 (two-model) for analyses at branch level, as well as the branch-site test of positive selection (Amodel vs. null model) [insert refs]. We tested different sets of hypotheses for each method (Table1).

Branch-models

To obtain an initial assessment of gene-wide positive selection, we used BUSTED (Branch-Site Unrestricted Statistical Test for Episodic Diversification).

We used codeml models 0 (one-model), 1 (free-model) and 2 (two-model) to test for the existence of different evolutionary patterns among coastal, riverine and oceanic dolphins. A likelihood-ratio test was performed to compare model 0 versus models 1 and 2.

Additionally, we used RELAX to explore the variation in the evolutionary rates across odontocete species from different habitats.

Branch-site models

To identify individual branches experiencing episodic diversifying selection on each gene, we used the branch-site analyses aBSREL

Site models

To test for differences in selective pressures at individual sites among sets of branches, we performed Contrast-FEL analysis with two sets of test branches, corresponding, respectively, to riverine and coastal dolphins.

Phenotype-genotype analyses

All hypotheses with signatures of positive selection (i.e. H2: river odontocetes and H4: coastal odontocetes) were further investigated for molecular convergence, using the PCOC (Profile Change One Change) toolkit (Rey et al., 2018) and for adaptive phenotype-genotype correlations, using PGBSM (Phenotype-Genotype Branch-Site Model) (Jones et al., 2020).

List of abbreviations

Declarations

Consent for publication

Availability of data and materials

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

LM and MFN conceived the study, designed the experiments and wrote the manuscript. LM retrieved, processed and analyzed the data. LF wrote the python scripts used to recover coding sequences from scaffold level genomes. AP wrote a bash script used to

simultaneously run multiple branch-site models on codeml. All authors contributed to the article and approved the submitted version.

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