Evolution of ion channels in cetaceans a natural experiment in the tree of life

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Abstract

Cetaceans could be seen as a natural experiment within the tree of life in which a mammalian lineage changed from terrestrial to aquatic habitats. This shift involved extensive phenotypic modifications representing an opportunity to explore the genetic bases of phenotypic diversity. Furthermore, the availability of whole genome sequences in representative species of all main cetacean groups means that we are in a golden age for this type of study. Ion channels are a crucial component of the cellular machinery for the proper physiological functioning of all living species. This study aims to explore the evolution of ion channels during the evolutionary history of cetaceans. To do so, we created a bioinformatic pipeline to annotate the repertoire of ion channels in the genome of the species included in our sampling. Our main results show that cetaceans have fewer ion channels than non-cetacean mammals and that the signal of positive selection was found in ion channels related to heart, locomotion, and hearing phenotypes. Interestingly the $Na_V1.5$ ion channel of most toothed whales (odontocetes) seems to be sensitive to TTX, similar to $Na_V1.7$, given the presence of tyrosine, instead of cysteine, in a specific position of the ion channel. Finally, the gene turnover rate of the cetacean crown group is more than two times faster than non-cetacean mammals.

Keywords: $Na_V1.5$, SCN5A, TTX, PKD1L1, gene turnover

Introduction

Understanding the genetic basis of diversity represents a central goal in evolutionary biology. The availability of whole-genome sequences in representative species of different groups represents a unique opportunity to advance this goal. Thus, the Tree of Life could be seen as a set of natural experiments that help us understand different evolutionary phenomena. For example, lineages have evolved traits of biomedical interest (e.g., cancer resistance), representing an opportunity to understand how the evolutionary process solves problems and translates their solution to us (Giroud et al. 2021; Tejada-Martinez et al. 2021; Tollis et al. 2021; Zhao et al. 2021; Oka et al. 2023; Thienel et al. 2023). Further, during the evolutionary history of vertebrates, colonizations of new habitats have resulted in severe phenotypic transformations, providing an opportunity to understand the genomic basis of diversity (McGowen et al. 2012; Nery, González, et al. 2013; Sun et al. 2013; M. Jiang et al. 2020; Bondareva et al. 2023).

The colonization of the aquatic environment by tetrapods occurred multiple times during their evolutionary history. Among mammals, the cetaceans (whales and dolphins) started transitioning from land to the sea during the Eocene around 50 Mya. A few million years later, the lineage entirely depended on the aquatic environment and diversified and occupied all the seas and many rivers of the world (Berta et al. 2005). The successful aquatic colonization from a terrestrial habitat demanded biological transformation due to gravity-related challenges, thermal regimes, a new pathogenic environment, different environmental stimuli that required sensory adaptations, and osmotic regulation, among others (Houssaye and Fish 2016; Kelley et al. 2016). Taking advantage of the advancement of genomic tools, these adaptations have been investigated from the molecular perspective to expand further our knowledge about the evolutionary process behind the conquest of an aquatic lifestyle (McGowen et al. 2011; McGowen et al. 2012; Nery, González, et al. 2013; Nery, Arroyo, et al. 2013; Sun et al. 2013; Nery et al. 2014; McGowen et al. 2020). Interestingly, several studies have reported the loss of genes as a strategy of phenotypic evolution (Feng et al. 2014; Nery et al. 2014; Sun et al. 2017; Huelsmann et al. 2019; Helsen et al. 2020; McGowen et al. 2020; Randall et al. 2022; Zheng et al. 2022; Osipova et al. 2023; Pinto et al. 2023).

Ion channels are integral membrane proteins that allow the passage of ions involved in a diverse repertoire of physiological processes. In the human and mouse genomes, 235 and 231 ion channels have been estimated, respectively, representing around 1% of the protein-coding genes of their genomes (Jegla et al. 2009). There is no doubt that ion channels represent a crucial part of the molecular machinery for the correct physiological functioning of living creatures. In fact, amino acid sequence variation has been linked to a wide range of pathological conditions, also called channelopathies (Ackerman and Clapham 1997; Ackerman 2004; Kim 2014). Thus, given their pivotal role in different physiological axes, some of which have diverged extensively in cetaceans due to the conquest of the aquatic environment, it seems interesting to study their evolutionary trend in this mammalian group.

This work aims to study the evolution of the repertoire of ion channels during the evolutionary history of cetaceans. To do so, we first created a bioinformatic pipeline to annotate the whole repertoire of ion channels in the genome of the species included in our sampling. After that, we estimated homologous relationships to study the role of positive selection and the variation in gene turnover rate. Our main results show 1) on average, cetaceans have fewer ion channels than non-cetacean mammals, 2) the signal of positive selection was found in ion channels related to heart, locomotion, and hearing phenotypes, all characteristics extensively modified in cetaceans, 3) the $Na_V1.5$ ion channel of toothed whales (odontocetes), other than species of the genus *Tursiops*, seems to be sensitive to TTX, similar to $Na_V1.7$, given a replacement of cysteine for a tyrosine, 4) the gene turnover rate of the cetacean crown group is more than two times faster in comparison to non-cetacean mammals.

Material and methods

Phylogenetic design, DNA sequences, and ion channel annotation

Our phylogenetic design included 15 mammalian species: five toothed whales (Odontoceti) (bottlenose dolphin, *Tursiops truncatus*; orca, *Orcinus orca*; beluga, *Delphinapterus leucas*; Yangtze river dolphin, *Lipotes vexillifer*; sperm whale, *Physeter catodon*), two baleen whales (Mysticeti) (common minke whale, *Balaenoptera acutorostrata*; blue whale, *Balaenaenoptera musculus*), two artiodactyls (cow, *Bos taurus*; pig, *Sus scrofa*), one carnivore (dog, *Canis familiaris*); one perissodactyl (horse,

Equus caballus); one chiroptera (microbat, *Myotis lucifugus*); one primate (human, *Homo sapiens*); one rodent (mouse, *Mus musculus*) and one proboscidean (African elephant, *Loxodonta africana*).

The protein-coding sequences (.faa file extension) for each species were downloaded from Ensembl v.105 (Yates et al. 2022) or the NCBI database (Sayers et al. 2022). In all cases, we kept the longest transcript for each gene. Of all the proteins present in the proteomes, we selected those that had between 2 and 35 transmembrane domains using the software TMHMM v2.0 (Krogh et al. 2001) (Fig. 1). We annotated the protein sequences based on the structural motifs using the program RPS-BLAST v2.13.0+ (with the option -outfmt 11) plus the rpsbproc package (Yang et al. 2020) (Fig. 1). We filtered the results based on the e-value threshold of 10^{-4} (Fig. 1). In parallel, we prepared a file containing the list of ion channel conserved domains based on the Conserved Domain Database (CDD) (Lu et al. 2020). We then compared this list with our RPS-BLAST v2.13.0+ plus the rpsbproc results using an *in-house* Perl script able to identify the ion channel repertoire for all sampled species (Fig. 1). The result was the list of ion channel protein sequences from each species for the next steps (Supplementary Table S1).

Homology inference

After having identified the ion channel repertoire from each sampled species, the next step was to infer homologous relationships. To do this, we used the program OMA standalone v2.5 (Altenhoff et al. 2021). We inferred Orthologous Groups (OGs) containing 1:1 orthologous genes and Hierarchical Orthologous Groups (HOGs) containing the set of genes that have descended from an ancestral gene. OGs were used to perform natural selection analyses by estimating the rate of non-synonymous (d_N) and synonymous (d_S) substitutions. In contrast, the HOGs were used to perform gene copy number variation analyses. Both analyses take into account the sister group relationships of the included species. Amino acid sequences were aligned using the FFT-NS-2 strategy of the program MAFFT v7.490 (Katoh and Standley 2013). Nucleotide alignments were obtained employing the amino acid alignments as templates using the software PAL2NAL (Suyama et al. 2006).

Molecular evolution analyses

To test for evidence of positive selection on the cetacean orthologous groups of ion channels, we estimated the ratio of the rate of non-synonymous (d_N) and synonymous substitutions (d_S) ($\omega=d_N/d_S$) using the program PAML v4.9 (Yang 2007). For each multiple sequence alignment, we compared models that allow ω to vary among codons (M1a vs. M2a, M7 vs. M8). M2a and M8 models allow a subset of sites to have $\omega > 1$, in contrast to the null model (M1a and M7) only includes site classes with $\omega < 1$. All nested models were compared using the Likelihood Ratio Test (LRT), and we applied the False Discovery Rate (FDR) as a multiple-test correction (Benjamini and Hochberg 1995).

Gene turnover rate analyses

To study variation in the gene turnover rate, we used the software CAFE v4.2.1(Han et al. 2013). Using this program, we estimated the rate of evolution (λ) and the direction of the change regarding the size of gene families across different lineages. We implemented two models, 1) in the first model, we estimated one λ value for cetaceans as a total group (crown and stem), and a second λ for all non-cetaceans branches of the tree, and 2) in the second model, we estimated one λ for the stem Cetacea, other λ for the crown Cetacea, and a third λ for all non-cetaceans branches of the tree. The divergence time between species was obtained from the TimeTree 5 database (Kumar et al. 2022)**.**

Enrichment analysis

To understand what molecular functions and phenotypes are associated with the genes that show the signature of positive selection, we used the web server Enrichr (Xie et al. 2021). This platform is a search engine that allows the querying of annotated genes combining knowledge from multiple databases to provide information regarding mammalian genes. We considered a category overrepresented if it had an adjusted probability value of less than 0.01.

Structural methods

Protein structure homology modeling was performed using the SWISS-MODEL server (https://swissmodel.expasy.org/) (Waterhouse et al. 2018). Structural figures were prepared using PyMOL Molecular Graphics System, Version 2.0.6 Schrödinger, LLC. Ligand-protein interaction diagrams were performed with LigPlot$^+$ v.2.2 (Laskowski and Swindells 2011).

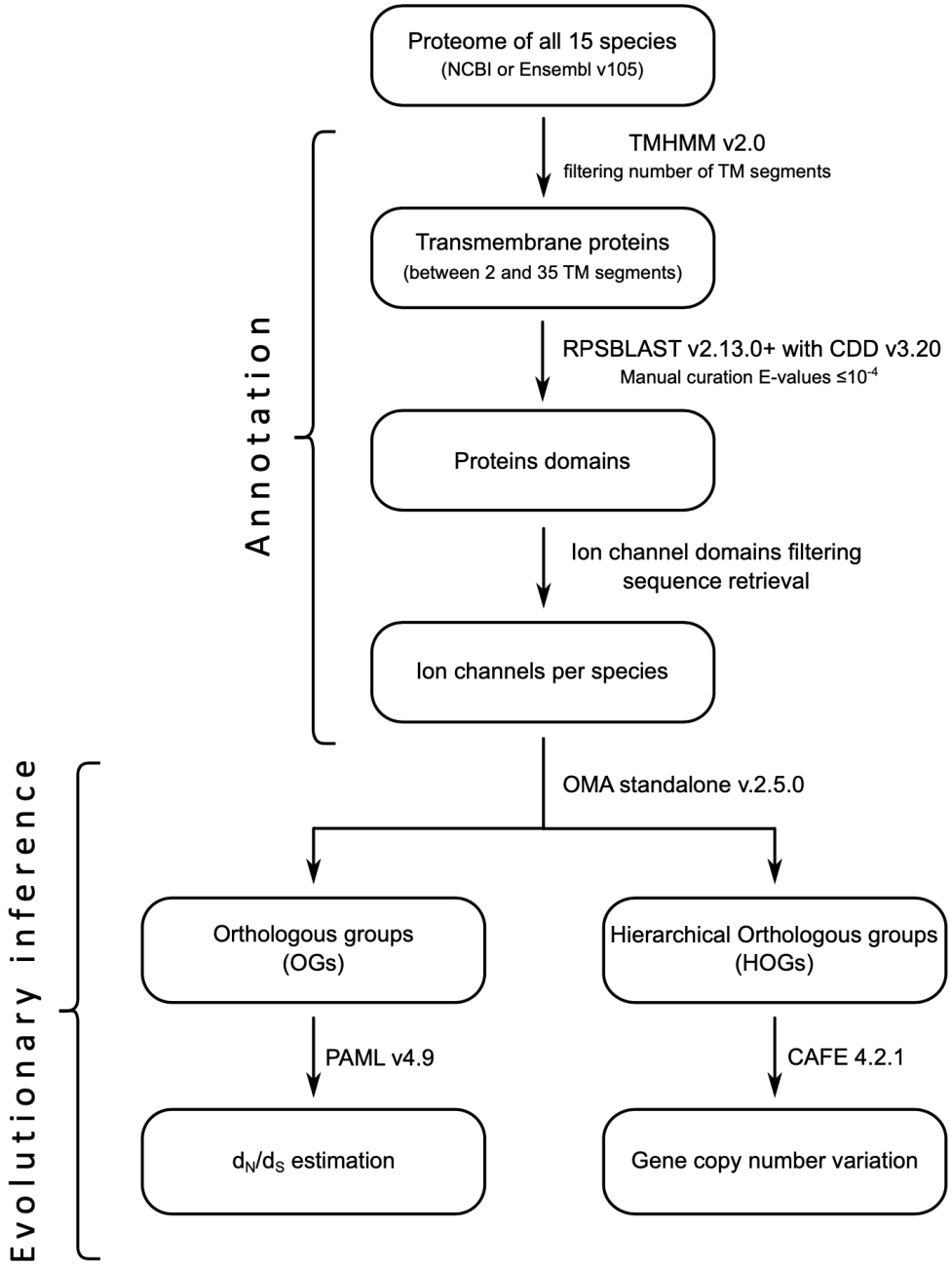


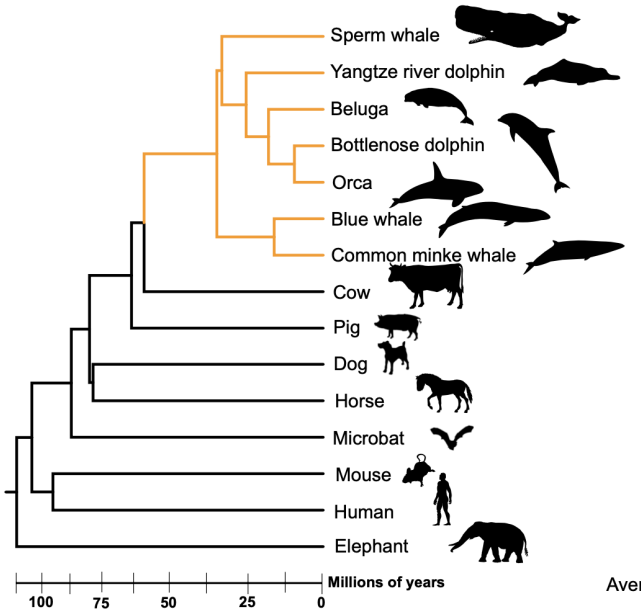
Figure 1. Flow diagram of the methodology used in this work. We divided the pipeline into two main steps, the annotation process, and the evolutionary inference. In the annotation process, we implemented tools like TMHMM (Krogh et al. 2001) and RPSBlast (Yang et al. 2020) to identify the repertoire of ion channels in the proteome of each species. Then, we applied the OMA standalone program to infer orthologous and hierarchical orthologous groups. After that, we used PAML (Yang 2007) to estimate the ratio of the rate of non-synonymous (d_N) and synonymous substitutions (d_S)($\omega=d_N/d_S$) and CAFE (Han et al. 2013) to study gene copy number variation. TM: Transmembrane

Results and Discussion

Ion channel annotation and homology inference

We annotated, on average, 208.93 ion channels in the genomes of the species included in our taxonomic sampling, representing 1.04% of the protein-coding genes (Fig. 2). The smallest number of ion channels (185) was obtained for the bottlenose dolphin (*Tursiops truncatus*), while the largest number (226) was in humans (*Homo sapiens*) (Fig. 2). Our results are comparable, although a little bit smaller than what is reported in the literature, 235 ion channels for humans (*Homo sapiens*) and 231 for the mouse (*Mus musculus*) (Jegla et al. 2009). Interestingly, on average, cetaceans possess fewer annotated ion channels than the non-cetacean mammals in our sampling (202.57 ± 11.57 vs. 214.50 ± 10.14, unpaired one-tailed t-test with d.f.=12.092; t-statistic = -2.109 and p-value = 0.028). This result is consistent with the hypothesis that gene loss can play a significant role in phenotypic evolution (Olson 1999; Albalat and Cañestro 2016; Helsen et al. 2020). Support for this idea has been widely documented in the literature for cetaceans and other groups (Feng et al. 2014; Nery et al. 2014; Sun et al. 2017; Huelsmann et al. 2019; Helsen et al. 2020; McGowen et al. 2020; Randall et al. 2022; Zheng et al. 2022; Osipova et al. 2023; Pinto et al. 2023).

According to the homology inference procedure, we obtained 115 orthologous groups in which all cetacean species were present. Additionally, 219 hierarchical orthologous groups containing two or more species were inferred.



	Protein-coding genes	Ion Channels	% Ion channels
Sperm whale	19717	196	0.99
Yangtze river dolphin	18890	210	1.11
Beluga	19091	193	1.01
Bottlenose dolphin	16550	185	1.12
Orca	19204	210	1.09
Blue whale	19518	206	1.06
Common minke whale	18832	218	1.16
Cow	21880	216	0.99
Pig	21303	224	1.05
Dog	20567	216	1.05
Horse	20955	221	1.05
Microbat	19728	199	1.01
Mouse	22469	214	0.95
Human	23500	226	0.96
Elephant	20033	200	1.00
Average	20129.13	208.93	1.04

Figure 2. Ion channel repertoire of the mammalian species included in our sampling. The first column reports the number of protein-coding genes of each species. The second column reports the number of ion channels annotated using the methodology previously described. Finally, the third column reports the proportion of ion channels in the proteome of each species. The cetacean clade is highlighted with orange branches. The divergence times were obtained from the Timetree 5 database (Kumar et al. 2022). Phylogenetic relationships were obtained from the literature (McGowen et al. 2019; Upham et al. 2019). Silhouette images were downloaded from PhyloPic (http://phylopic.org/).

Ion channels with the signature of positive selection are related to heart, locomotion, and hearing physiology

After performing an enrichment analysis of the genes with the signal of positive selection against the mammalian phenotype ontology database (Eppig et al. 2015), we recovered 69 enriched categories with an adjusted p-value < 0.01 (Supplementary Table S2). These categories are related to phenotypes linked to the group of genes that are well known to have been modified due to the conquest of the aquatic environment (e.g., circulatory, locomotor, and auditory systems, among others). Comparable results have been obtained in previous studies where groups of genes related to specific characteristics are studied (Tollis et al. 2019; Tejada-Martinez et al. 2021; Silva et al. 2023), also in genome-wide studies (McGowen et al. 2012; Nery, González, et al. 2013; Sun et al. 2013; Park et al. 2015).

Our study found that the most frequent category was related to heart physiology. We identified categories such as “decreased heart rate”, “sinus bradycardia”, and “decreased cardiac muscle contractility”, which align with previous literature on changes in heart function during diving (Table 1) (Scholander 1940; Irving et al. 1941; Williams et al. 2015; Goldbogen et al. 2019). One of the main challenges for aquatic living is coping with the acute hypoxia that occurs during extended breath-holding. To overcome the physiological effects of deep dives, remarkable adaptations have evolved, including significant decreases in heart rate and redistribution of blood flow to essential aerobic tissues (Kooyman and Ponganis 1998). Although the basic structure of the cetacean heart is similar to that of other mammals, our findings emphasize the importance of ion channel genes in adapting to diving. Thus, besides the well-documented morphological changes in the cetacean heart (Tarpley et al. 1997; Latorre et al. 2022) mostly attributed to diving adaptations, our results show adaptive changes in genes encoding ion channels, which are fundamental for their physiological divergence.

Among the associated genes related to heart physiology in cetaceans (Table 1), the Sodium Voltage-Gated Channel Alpha Subunit 5 (SCN5A) is the most frequent. The SCN5A gene encodes the alpha subunit of the sodium channel $Na_V1.5$, mainly expressed in the heart (Uhlen et al. 2010). This protein mediates the voltage-dependent sodium ion permeability of the cardiac muscle, playing an essential role in regulating cardiac electrophysiological function (Li et al. 2018). In addition, mutations in this ion channel cause a broad range of electrical disorders and structural abnormalities in humans (Li et al. 2018; D. Jiang et al. 2020; Rivaud et al. 2020). Past studies have also found this ion channel under positive selection in cetaceans (Sun et al. 2013).

Table 1. Enriched categories related to heart physiology in cetaceans based on the MGI mammalian phenotype ontology database using the Enrichr platform (https://maayanlab.cloud/Enrichr/).

Category	p-value	Adjusted p-value	Associated Genes
Atrial fibrillation	5.14E-07	4.81E-05	RYR2; CACNA1D; SCN5A
Abnormal heart rate	8.76E-06	3.76E-04	HCN4; CACNA1D; SCN5A
Atrioventricular block	1.03E-05	3.76E-04	RYR2; CACNA1D; SCN5A
Abnormal myocardial fiber physiology	1.07E-05	3.76E-04	RYR2; CACNA1D; CACNA1C; SCN5A
Decreased heart rate	1.64E-05	5.42E-04	HCN4; RYR2; CACNA1D; CACNA1C; SCN5A
Abnormal cardiac muscle contractility	2.33E-05	6.87E-04	RYR2; CACNA1C; SCN5A
Decreased cardiac muscle contractility	1.11E-04	2E-03	HCN4; RYR2; CACNA1C; SCN5A
Irregular heartbeat	1.23E-04	2E-03	RYR2; CACNA1C; SCN5A
Prolonged PR interval	2.01E-04	3E-03	CACNA1D; SCN5A; CACNA1F
Sinus bradycardia	2.45E-04	3E-03	CACNA1D; SCN5A
Shortened QRS complex duration	3.47E-04	4E-03	CLCNKA; SCN5A
Ventricular premature beat	3.47E-04	4E-03	RYR2; SCN5A
Ventricular tachycardia	4.04E-04	5E-03	RYR2; SCN5A
Abnormal sinoatrial node conduction	5.32E-04	5.63E-03	CACNA1D; SCN5A
Abnormal heart electrocardiography waveform feature	9.24E-04	8.10E-03	RYR2; SCN5A

In the case of the $Na_V1.5$ ion channel, several amino acid polymorphisms related to pathological conditions associated with abnormal heart rhythm have been described (Li et al. 2018; Rivaud et al. 2020). This knowledge represents an opportunity to make a screening in non-model species, especially those in which the heart rhythm is different in comparison to humans. To do so, we compared the positions for which amino acid polymorphisms have been described in humans in the cetacean species included in our study. Our analysis found that most species have the most common alleles described for humans (not shown). However, there are some positions for which cetaceans have

the amino acid variant associated with a pathological condition (Table 2). For example, we found five positions where all the cetacean species possess the variant associated with a disease (Table 2). In other cases, we found some species having pathological variants (Table 2). In one case, all cetacean species have a gap in the alignment (Table 2, position 681). Although specific polymorphisms in this ion channel are associated with a diversity of diseases (Li et al. 2018; Rivaud et al. 2020), our results depict that most of the positions in which pathological variants are present in cetaceans are related to the long QT syndrome 3 disease (LQT3; MIM: 603830; Table 2). In this disease, and during repolarization, the myocardial cell experiences continuous sodium influx due to an abnormal sodium channel. This is because the ion channel fails to achieve complete inactivation, preventing the expected cessation of sodium entry (Zareba et al. 1998; Wallace et al. 2019). At the physiological level, LQT3 is mainly characterized by marked resting bradycardia (Zareba et al. 1998; Wallace et al. 2019). The heart regular rhythm disruption has been reported in the other two associated diseases, Brugada syndrome (BRGDA1; MIM: 601144) and Atrial fibrillation familial 10 (ATFB10; MIM: 614022). Future studies should be directed at understanding the biophysical properties of the cetacean $Na_V1.5$ channel. As a whole, mutations in a gene affecting the rhythm of the heartbeat make sense in the light of the adaptations of the heart physiology of cetaceans which are characterized, among other things, by a decreased heart rate during foraging dives and tachycardia events at the sea surface as they recovered from its oxygen debt (Kooyman and Ponganis 1998; Goldbogen et al. 2019).

Although the $Na_V1.5$ and $Na_V1.7$ sodium channels have similar selectivity filters (Shen et al. 2019; D. Jiang et al. 2020), the $Na_V1.5$ channel has an affinity about two orders of magnitude lower for tetrodotoxin (TTX) (Sunami et al. 2000; Walker et al. 2012), a sodium channel blocker that is found in a variety of marine animals (e.g., pufferfish, frogs, horseshoe crabs, blue-ringed octopus, gastropods, starfish, among others) (Jal and Khora 2015). This difference is due to a single amino acid substitution, where the human $Na_V1.5$ channel has a cysteine at position 373 instead of a tyrosine (Fig. 3). As expected, all $Na_V1.7$ channels of cetaceans possess a tyrosine amino acid at a structurally related position, Y362 in human $Na_V1.7$ (Fig. 3), making it susceptible to TTX blockage. Interestingly, besides the bottlenose dolphin, the $Na_V1.5$ channel of the

toothed whales (Odontoceti) species included in our study possesses a tyrosine instead of cysteine (Fig. 3), making it potentially sensitive to TTX. Furthermore, three-dimensional protein structure modeling of sperm whale $Na_V1.5$ and $Na_V1.7$ shows a conserved spatial arrangement of amino acid residues that are important for TTX binding on human $Na_V.7$, including Y362 that establishes a π-cation interaction with the guanidinium group of TTX, an interaction that is not possible by C373 of human $Na_V1.5$ (Fig. 4). To strengthen our conclusions, we retrieved additional $Na_V1.5$ sequences from toothed whales to see if they also have tyrosine instead of cysteine. In our new searches, we recovered the $Na_V1.5$ ion channel of the long-finned pilot whale (*Globicephala melas*), Pacific white-sided dolphin (*Lagenorhynchus obliquidens*), Narwhal (*Monodon monoceros*), Yangtze finless porpoise (*Neophocaena asiaeorientalis*), and vaquita (*Phocoena sinus*). We found a tyrosine residue in all cases, providing further support to our results (Fig. 3). We also found a different species of the genus *Tursiops*, the Indo-Pacific bottlenose dolphin (*Tursiops aduncus*), and following the trend we are describing, it has a cysteine residue (Fig. 3). Finally, to be sure that this cysteine to tyrosine replacement only occurred in toothed whales, we also retrieved additional $Na_V1.5$ sequences from baleen whales (Mysticeti). We added two more species, the humpback whale (*Megaptera novaeangliae*) and the gray whale (*Eschrichtius robustus*). Both species have a cysteine residue, confirming our initial conclusion (Fig. 3). Thus, the amino acid substitution from cysteine to tyrosine occurred in the last common ancestor of toothed whales (Odontoceti), which lived between 34 and 33 million years ago (Kumar et al. 2022). However, in the last common ancestor of the genus *Tursiops*, it was reversed (Fig. 3). This is interesting because documentarists have been able to record dolphins "playing" with pufferfish, with the threat of being exposed to TTX that the pufferfish releases when it feels threatened. However, since the toxin is released into the water, it seems to be not in lethal doses, and it has been proposed it could cause a trance-like state. This could be possible because pufferfish mainly accumulate TTX in the skin (Zhang et al. 2020), especially when they are young (Tatsuno et al. 2013), an organ in direct contact with the mouth of the dolphin. In fact, it has been observed that dolphins behave differently after "playing" with the pufferfish. Therefore, this behavior could be related to the reversal in the amino acid substitution,

which makes it plausible that the $Na_V1.5$ ion channel of the species of the genus *Tursiops* has a lower affinity for TTX, like all mammals. Thus, besides having mutations associated with human channelopathies, the $Na_V1.5$ channel of a subset of cetaceans, toothed whales (Odontoceti), is predicted to have a higher affinity to TTX than other mammals. Further lab experiments are the next logical step to reveal the functional divergence of the $Na_V1.5$ in this particular group of mammals.

The Ryanodine Receptor 2 (RYR2) gene, the second most frequent gene in Table 1, encodes for a protein that is one of the components of the largest ion channel known up to date. It is mainly expressed in the heart (Uhlen et al. 2010), and its primary function is controlling Ca^{+2} release from the sarcoplasmic reticulum throughout the cardiac cycle (Fowler and Zissimopoulos 2022). Mutations in this channel have been associated with Arrhythmogenic Right Ventricular Dysplasia, familial, 2 (ARVD2), Ventricular tachycardia, catecholaminergic polymorphic, 1 (CPVT1), Ventricular arrhythmias due to cardiac ryanodine receptor calcium release deficiency syndrome (VACRDS), among others (Sleiman et al. 2021). However, we did not find a case for this ion channel in which cetacean species possess pathological variants.

Table 2. Summary of the $Na_V1.5$ non-synonymous polymorphisms among cetaceans. One letter-coded amino acids matching the rare alleles in humans are in bold. Dashes indicate a gap in the multiple sequence alignment. Variant IDs are taken from the UniProtKB database.

Position	53	526	579	639	681	689	997	1114	1951	2004	
Common allele / Pathologic Allele	R / Q	R / H	G / R	G / R	H / P	R / H	A / T	D / N	V / M	F / V	F / L
Variant ID	Var_074698	Var_074358	Var_074361	Var_03664	Var_026359	Var_074374	Var_074405	Var_009935	Var_055219	Var_074484	Var_055221
Bottlenose dolphin	**Q**	**H**	G	**R**	-	R	**T**	D	**M**	I	
Blue whale	**Q**	**H**	G	**R**	-	**H**	**T**	D	**M**	I	
Minke whale	**Q**	**H**	G	**R**	-	R	**T**	D	**M**	I	
Beluga whale	**Q**	**H**	G	**R**	-	R	**T**	D	**M**	I	
Orca	**Q**	**H**	**R**	**R**	-	R	**T**	D	**M**	I	
Sperm whale	**Q**	**H**	G	**R**	-	R	**T**	N	**M**	I	
Yangtze River dolphin	**Q**	**H**	G	**R**	-	R	**T**	D	**M**	**V**	
Disease	LQTR3	BRGDA1	LQTR3	LQTR3	BRGDA1	LQTR3	BRGDA1	LQTR3	ATFB10	LQTR3, BRGDA1	

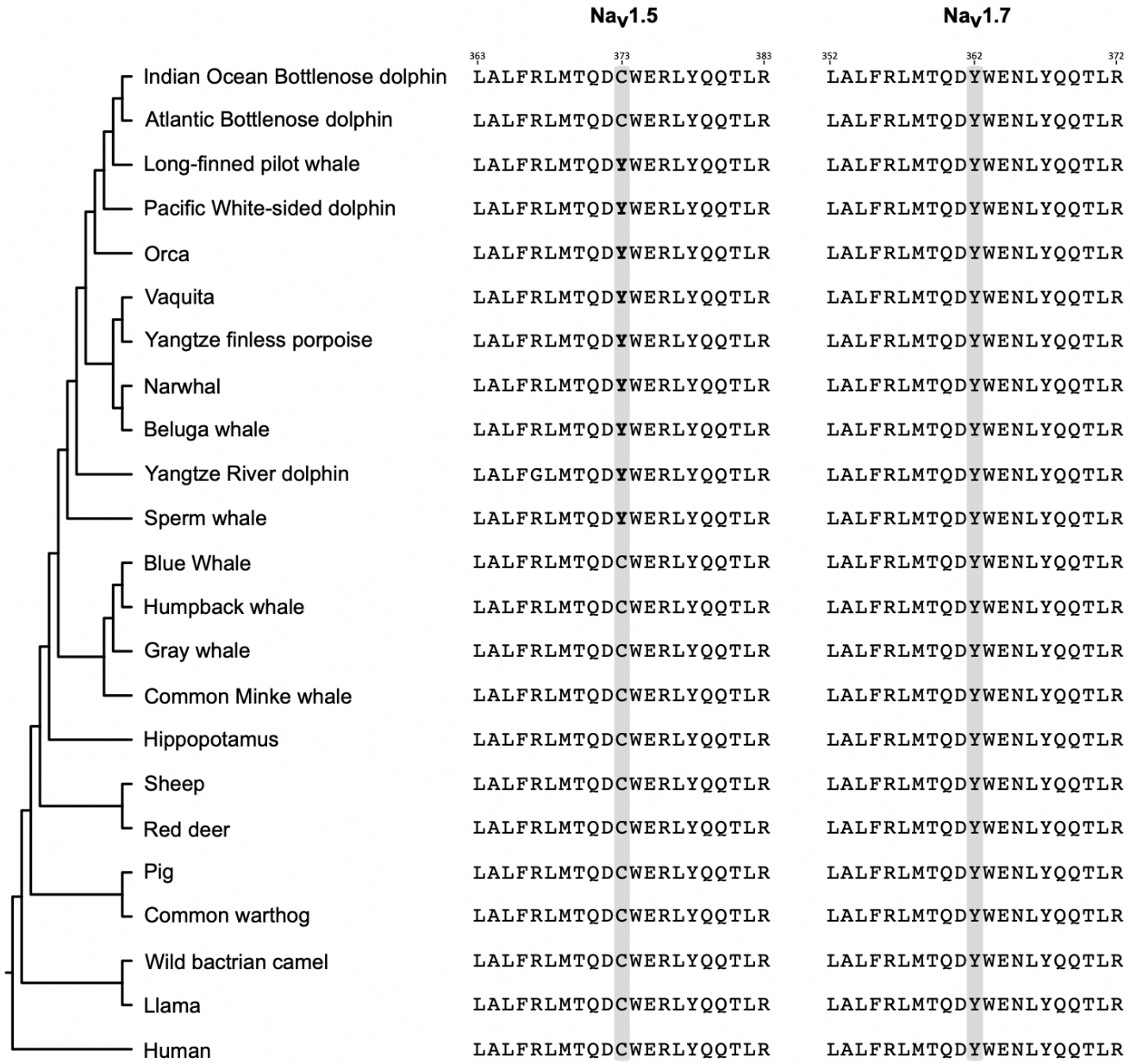


Figure 3. Amino acid alignment of a segment between the transmembrane segments 5 and 6 of the $Na_V1.5$ and $Na_V1.7$ ion channels containing the amino acid positions responsible for the sensitivity to TTX (shaded in gray). The numbering of the amino acids is with respect to the human sequences ($Na_V1.7$, NM_001365536.1; $Na_V1.5$, NM_001099404.2). Phylogenetic relationships were obtained from the literature (McGowen et al. 2019; Upham et al. 2019). In bold are the amino acids tyrosine (Y) in the $Na_V1.5$ ion channels of toothed whales (Odontoceti), which would make them more sensitive to TTX.

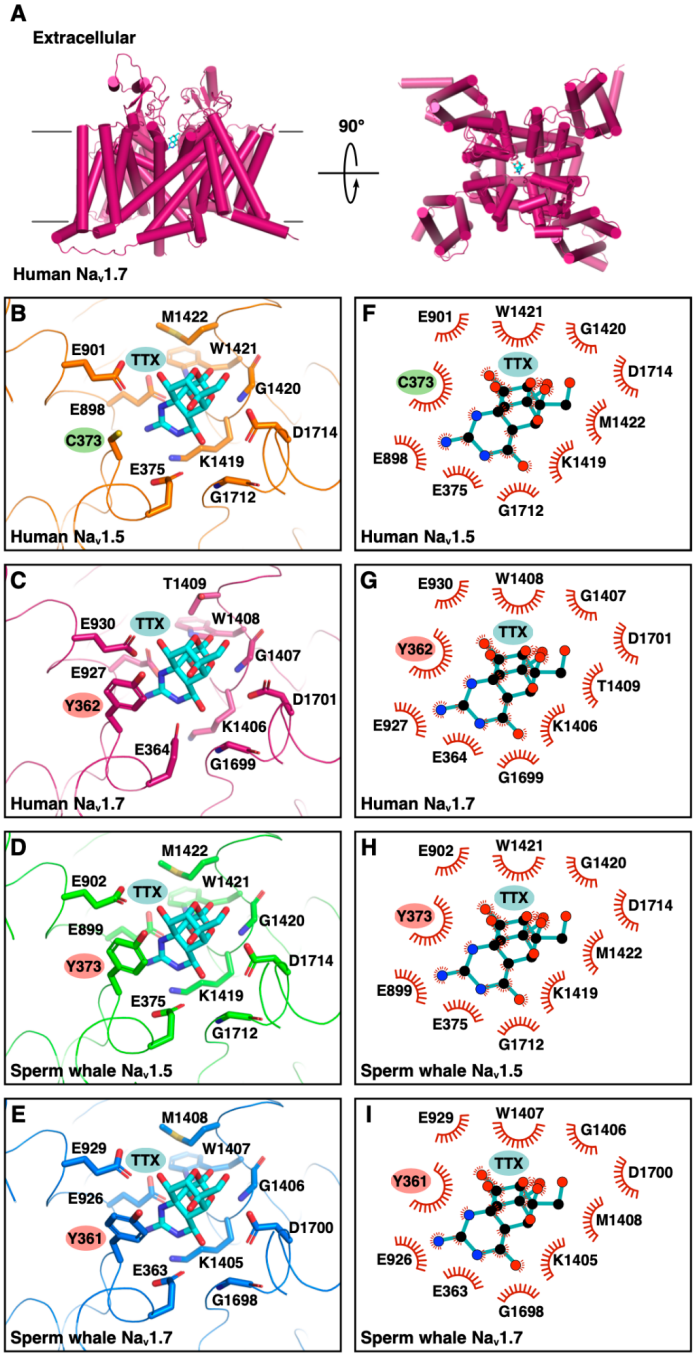


Figure 4. Comparison of the binding site for TTX in human and sperm whale $Na_V1.5$ and $Na_V1.7$. A) Cartoon representation (alpha-helices shown as cylinders) of human $Na_V1.7$ colored magenta, and stick model of TTX with carbon atoms colored cyan (PDB code 6J8I showing only chain B and TTX). B-E) Ribbon representation of the TTX binding site on the indicated sodium channels, and stick representation of sodium channel TTX binding site amino acid residues with carbon atoms colored orange (B; human $Na_V1.5$; PDB code 6LQA), magenta (C; human $Na_V1.7$; PDB code 6J8I), green (D; sperm whale $Na_V1.5$; model generated by SWISS-MODEL), or blue (E; sperm whale $Na_V1.7$; model generated by SWISS-MODEL), and of TTX with carbon atoms colored cyan. Highlighted are the positions of C373 in human $Na_V1.5$, of Y362 in human $Na_V1.7$ which is critical for binding via a π-cation interaction with the

1,2,3-guanidinium group of TTX, and of Y373 and Y361 of sperm whale $Na_V1.5$ and $Na_V1.7$, respectively, predicted to interact with TTX via a similar p-cation interaction. The carbonyl and amino groups of glycine residues are depicted for better visualization. F-I) Two- dimensional, schematic representation of the position of TTX shown in B-E using LigPlot$^+$.

We also retrieved categories related to locomotion (Table 3), one of the phenotypes significantly modified in cetaceans, with profound changes in the body plan due to the conquest of the aquatic environment. Unlike terrestrial mammals, cetaceans have elongated bodies and absent hindlimbs. The forelimb was modified into a flipper, and vertical movements of the tail accomplished locomotion. Moreover, moving into the water column imposes a highly energetic cost requiring a greater force than moving into the terrestrial environment, which translates into a need for stronger muscle contraction (Berta et al. 2005). In stronger muscle contraction, ion channels play a fundamental role by initiating the influx of ions, including sodium transport, into the cells. This result agrees with Sun et al. (2013), which reported the signature of positive selection in genes related to motor activity and muscle contraction in cetaceans. The two most frequently mentioned ion channels in Table 3 are the Calcium Voltage-Gated Channel Subunit Alpha1 A (CACNA1A) and Glycine Receptor Alpha 1 (GLRA1). In neither case, we found pathological variants in cetaceans. Thus, like the heart case previously described, given that marine mammal muscle architecture is similar to most other mammals (Würsig et al. 2017), the fact that we recovered several ion channels related to locomotion highlights their fundamental role in physiological divergence.

Table 3. Enriched categories related to locomotion in cetaceans based on the MGI mammalian phenotype ontology database using the Enrichr platform (https://maayanlab.cloud/Enrichr/).

Category	p-value	Adjusted p-value	Associated Genes
Abnormal motor capabilities/coordination/movement	7,35E-10	1,03E-07	TMC1; GLRA1; SCN8A; KCNMA1 ;CACNA1A; CACNA1C; CLCN3; CACNA1E
Seizures	2,09E-07	2,35E-05	GLRA1; SCN8A; CACNA1A; TRPM7; SCN5A; CLCN3
Abnormal locomotor behavior	1,10E-06	7,24E-05	GRIA1; SCN8A; KCNMA1; CACNA1B; CACNA1A; KCNK2

Abnormal gait	1,52E-06	8,55E-05	GRIA1; TMC1; GLRA1; SCN8A; KCNMA1; CACNA1B; CACNA1A; CLCN3
Tremors	2,49E-05	6,95E-04	GLRA1; SCN8A; KCNMA1; CACNA1A; TRPM7; CLCN3
Abnormal posture	2,60E-05	6,95E-04	GLRA1; SCN8A; CACNA1A; CLCN3
Increased susceptibility to pharmacologically induced seizures	5,78E-05	1,E-03	RYR2; GLRA1; CACNA1A; KCNK2
Impaired coordination	1,50E-04	2,E-03	GLRA1; SCN8A; KCNMA1; CACNA1A; CACNA1C; CLCN3
Short stride length	2,25E-04	3,E-03	GLRA1; KCNMA1; CACNA1A
Tonic-clonic seizures	2,25E-04	3,E-03	RYR2; GLRA1; CLCN3
Dystonia	7,54E-04	7,30E-03	SCN8A; CACNA1A
Muscle spasm	1,21E-03	9,83E-03	GLRA1; CACNA1A

The senses of cetaceans have radically changed in association with aquatic living. Hearing is undoubtedly the most critical sense for life underwater. In fact, the cetacean hearing has evolved to be remarkable. For example, toothed whales (Odontoceti) have developed sophisticated high-frequency hearing with the evolution of echolocation, defined as emitting sound signals and perceiving their surroundings through the returned echoes (Morisaka 2012). On the other hand, baleen whales (Mysticeti) are specialized in low-frequency hearing, suitable for long-distance communication (Edds-Walton 1997). Differently from the circulatory and muscular systems mentioned before, the underwater hearing evolution involved many anatomical changes, and the auditory apparatus of terrestrial mammals and cetaceans are very different (Berta et al. 2005). Accordingly, molecular adaptation in many hearing-related genes and pathways has been reported in the cetacean lineage (Li et al. 2010; Liu et al. 2018; McGowen et al. 2020). These genes are expressed in the outer hair cells, which are crucial for high-frequency hearing. For example, TMC1, one of the positively selected genes found here, was documented to evolve convergently between toothed whales (Odontoceti) and echolocating bats, highlighting the importance of this gene to high-frequency hearing (Parker et al. 2013; Liu et al. 2018). According to our results, the most frequent gene in Table 4 is the Voltage-dependent L-type calcium channel subunit

alpha-1D (CACNA1D), which mediates the entry of calcium ions into excitable cells and is coincidently related to impaired cardiac conduction and deafness (Baig et al. 2011). In this case, we did not find pathological variants in cetaceans either.

Table 4. Enriched categories related to the auditory system in cetaceans based on the MGI mammalian phenotype ontology database using the Enrichr platform (https://maayanlab.cloud/Enrichr/).

Category	p-value	Adjusted p-value	Associated Genes
Cochlear outer hair cell degeneration	2.12E-05	6.62E-04	TMC1; KCNMA1; KCNQ4; CACNA1D
Absent distortion product otoacoustic emissions	4.40E-05	1E-03	KCNMA1; KCNQ4; CACNA1D
Sensorineural hearing loss	5.77E-05	1E-03	KCNMA1; KCNQ4; CACNA1D
Abnormal cochlear IHC afferent innervation pattern	2.01E-04	3E-03	KCNMA1; CACNA1D
Abnormal cochlear inner hair cell physiology	2.94E-04	4E-03	KCNMA1; CACNA1D
Abnormal cochlear outer hair cell physiology	2.94E-04	4E-03	KCNQ4; CACNA1D
Increased or absent threshold for auditory brainstem response	8.83E-04	8.10E-03	TMC1; SCN8A; KCNQ4; CACNA1D

Accelerated gene turnover rate in cetaceans

The availability of whole-genome sequences have revealed that variation in gene copy number is abundant in nature (Schrider and Hahn 2010) and related to the origin of phenotypic diversity. For example, a survey of more than 9,000 gene families in primates suggested that humans possess faster gene turnover than other mammals (Hahn et al. 2007). In this study, the authors found several expansions (e.g., centaurin gamma gene family) in the lineage leading to humans that are related to the unique attributes of our species (e.g., enlarged brains) (Hahn et al. 2007), establishing a link between copy number variation and evolutionary innovations.

In our results, we also found variation in the gene turnover rate (Fig. 5). The model estimating different λ parameters for cetaceans, as a total group and non-cetacean mammals, showed that the first group possesses a rate of evolution (λ_C = 0.0021) 2.33 times faster compared to non-cetacean mammals (λ_o = 0.0009; Fig. 5). In the second model the estimated λ parameter for the crown group cetacea (λ_C = 0.0024)

was 2.6 times faster in comparison to non-cetacean mammals (λ_0 = 0.0009) and 8 times faster than the value estimated for the last common ancestor of cetaceans (λ_{anc} = 0.0003) (Fig. 5), suggesting that most of the variation in gene copy number occurred during the radiation of the group. Similar results were obtained when tumor suppressor genes were analyzed (Tejada-Martinez et al. 2021).

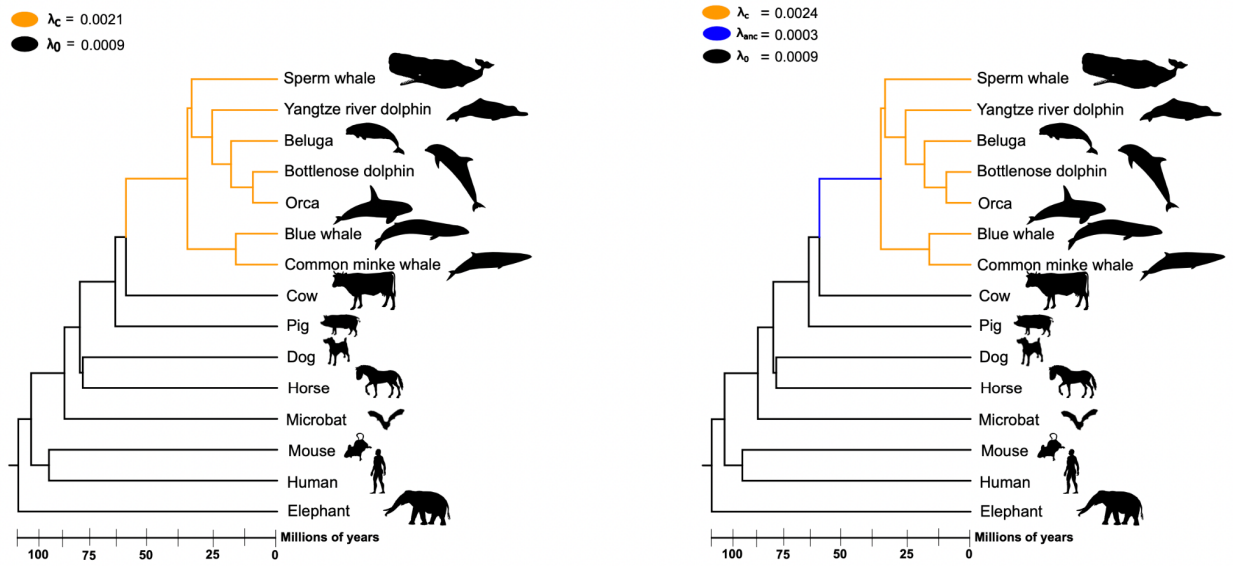


Figure 5. Gene turnover rates of ion channels. The first model (left panel) estimated the rate of evolution (λ) of ion channels for cetaceans as a total group (orange branches) and for non-cetacean mammals (black branches). Under this model, the λ value for cetaceans is more than two times faster than non-cetacean mammals. The second model (right panel) estimated λ values for the last common ancestor of cetaceans (blue branch), for the crown group cetacea (orange branches), and for non-cetacean mammals (black branches).

According to our estimates, the number of copies in cetaceans varies between zero and 13. We found four hierarchical orthologous groups in which all cetacean species have no copies (ASIC5, TMEM235, KCNMB3, and PKD1L1). In these cases, we double-checked the information and found different situations. In the case of the acid-sensing ion channel 5 (ASIC5), we found it in the fasta file containing all protein-coding genes of baleen whales (Mysticeti), but it was predicted to have less than 2 transmembrane segments, so it was not included in the next step in our pipeline. A similar situation occurred for the transmembrane protein 235 (TMEM235) and calcium-activated potassium channel subunit beta-3 (KCNMB3) genes. The polycystic kidney disease protein 1-like 1 (PKD1L1) gene was only found in Orca, but it was not

predicted to have any transmembrane segment. We also noticed that the PKD1L1 sequence associated with the accession number (XP_033267232.1) was removed from NCBI. According to ENSEMBL, no cetacean species was predicted to have an ortholog of the human PKD1L1 gene. Further, a comparison of the genomic region of the human (*Homo sapiens*), which possesses the PKD1L1 gene, with the corresponding chromosomal region in the sperm whale (*Physeter macrocephalus*), common minke whale (*Balaenoptera acutorostrata*), and vaquita (*Phocoena sinus*) suggests that the PKD1L1 gene is not present in the cetacean lineage (Fig. 6). It is worth noting that traces of the PKD1L1 gene are present in the sperm whale and vaquita genomes (Fig. 6). In figure 6 we also included the comparison with the horse (*Equus caballus)*, a species that share a common ancestor with humans at the same age as cetaceans, to show the conservation pattern of the chromosomal region harboring the PKD1L1 gene (Fig. 6). So, it is highly probable that this gene is not present in the cetacean genome. This result agrees with the study of Turakhia et al. (2020); however, they also show that gene loss is not a cetacean-specific evolutionary event, as they did not find the PKD1L1 gene in other cetartiodactyla species (e.g., alpaca, Bactrian camel, goat, sheep, Tibetan antelope, and cow) (Turakhia et al. 2020). In agreement, in our PKD1L1 hierarchical orthologous group, the cow and the pig were absent. Thus, this result suggests that the deletion of the PKD1L1 gene occurred in the last common ancestor of cetartiodactyla. PKD1L1 is a member of the TRP gene family, mainly expressed in the testis and heart (Yuasa et al. 2002). It also has functions related to establishing left-right asymmetry in positioning and patterning internal organs and associated vasculature by forming heteromeric channels with PKD2, functioning as sensors of the nodal flow (Pennekamp et al. 2002; Field et al. 2011; Norris 2012; Grimes et al. 2016; Esarte Palomero et al. 2023). However, this gene loss does not translate into functional consequences in cetaceans and even-toed ungulates, probably due to a certain degree of redundancy that could serve as a backup with functionally overlapping family members (Nowak et al. 1997; Félix and Barkoulas 2015; Albalat and Cañestro 2016).

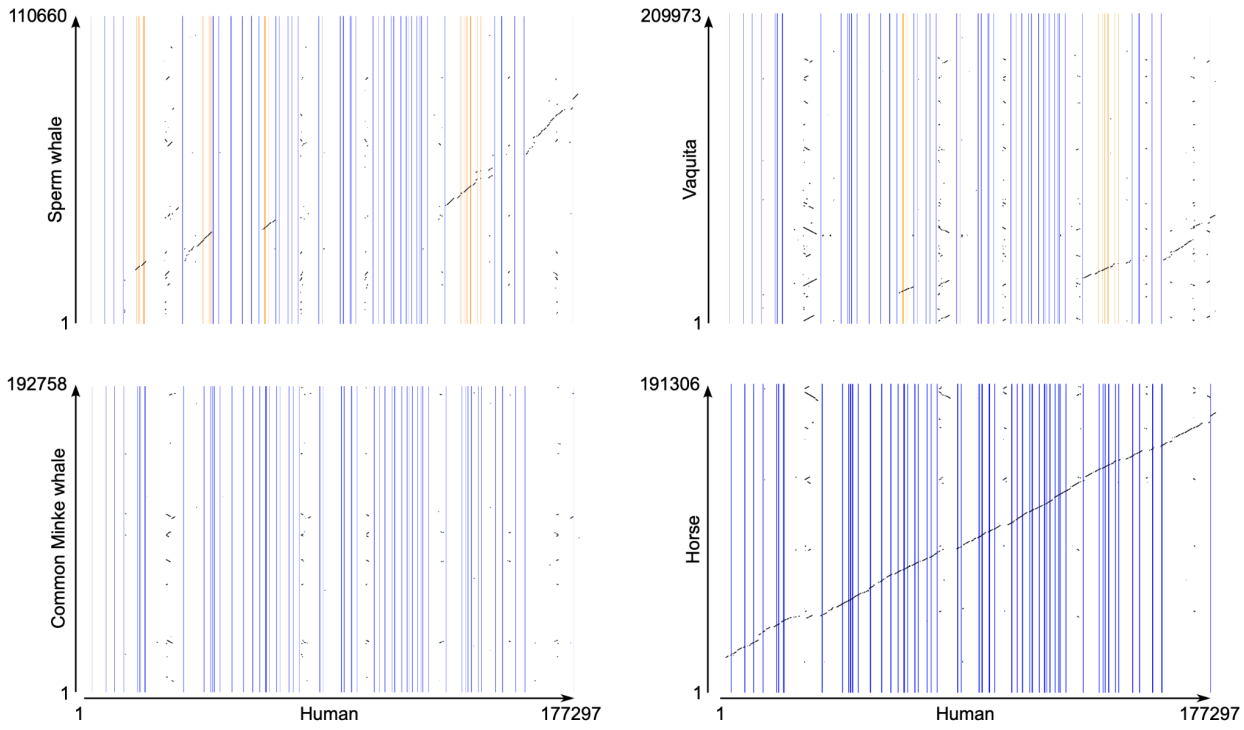


Figure 6. Pairwise dot-plot comparison of the genomic region containing the polycystic kidney disease protein 1-like 1 (PKD1L1) gene of the human (*Homo sapiens*) with the corresponding region in the sperm whale (*Physeter macrocephalus*), common minke whale (*Balaenoptera acutorostrata*), vaquita (*Phocoena sinus*), and horse (*Equus caballus*). Vertical lines denote exons, and regions in between are introns. Orange vertical lines indicate exons that are still present in the species that lost the PKD1L1 gene.

The hierarchical orthologous group that contained the highest number of copies corresponded to the transmembrane protein 37 (TMEM37), a gamma subunit of voltage-gated calcium channels, with 13 copies. In the case of the non-cetacean mammals in our sampling, this hierarchical orthologous group has six copies, suggesting that the 13 copies represent a cetacean-specific expansion. After that hierarchical orthologous group with 13 gene copies, there are three groups with nine copies (P2RX4, KCNK13, and CLCN4). In all cases, the non-cetacean mammals in our sampling have fewer copies than cetaceans, 5, 8, and 8, respectively.

A more detailed assessment of the hierarchical orthologous groups provides a better panorama of the evolutionary trend in which cetacean species possess fewer ion channels than the non-cetacean mammals in our sampling. Our analysis found that out of the 219 hierarchical orthologous groups inferred, cetaceans possess more gene copies in 23. On the other hand, in 42 hierarchical orthologous groups, both groups

have the same number of gene copies. At the same time, in 154 hierarchical orthologous groups, non-cetacean mammals possess more copies than cetaceans. Thus, our results agree with other studies showing that gene loss played a significant role in the conquest of the aquatic habitat by cetaceans.

Conclusions

In this work, we designed a bioinformatic pipeline that identifies the entire repertoire of ion channels of any species. In our case, we used it to study the evolution of ion channels in cetaceans, a mammalian group that, due to the conquest of the aquatic environment, has extensively modified physiological axes in which ion channels play a significant role. Our results indicate that cetaceans possess fewer ion channels than terrestrial mammals, consistent with the difference in gene turnover rate estimated for the group. Furthermore, most of the genes with the signal of positive selection are related to heart, locomotion, and hearing phenotypes, consistent with previous studies. Interestingly, the $Na_V1.5$ ion channel of cetaceans possesses amino acid variants identified as pathological in humans. The $Na_V1.5$ channel of mammals is about two orders of magnitude less sensitive to TTX than NaV1.7. This difference is due to a cysteine residue instead of a tyrosine in a specific channel position. However, our work shows that most species of toothed whales (Odontoceti) possess a tyrosine amino acid residue in that particular position of the $Na_V1.5$ channel, making them potentially sensitive to TTX, similar to NaV1.7. However, it is important to recall that the effect of an amino acid replacement depends on the genetic background. So, future studies should be directed to evaluate the biochemical/biophysical performance of the $Na_V1.5$ channel of toothed whales, especially their sensitivity to TTX. Finally, the natural experiment that cetaceans represent in the Tree of Life provides an excellent model to advance our understanding of the genetic bases of phenotypic diversity. The number of genomes that exist today and those that are yet to come will make the field of evolutionary genomics a significant contributor to different disciplines in biology and medicine.

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