



Research paper

Into the Blue: Exploring genetic mechanisms behind the evolution of baleen whales

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ABSTRACT

Marine ecosystems are ideal for studying evolutionary adaptations involved in lineage diversification due to few physical barriers and reduced opportunities for strict allopatry compared to terrestrial ecosystems. Cetaceans (whales, dolphins, and porpoises) are a diverse group of mammals that successfully adapted to various habitats within the aquatic environment around 50 million years ago. While the overall adaptive transition from terrestrial to fully aquatic species is relatively well understood, the radiation of modern whales is still unclear. Here high-quality genomes derived from previously published data were used to identify genomic regions that potentially underpinned the diversification of baleen whales (Balaenopteridae). A robust molecular phylogeny was reconstructed based on 10,159 single copy and complete genes for eight mysticetes, seven odontocetes and two cetacean outgroups. Analysis of positive selection across 3,150 genes revealed that balaenopterids have undergone numerous idiosyncratic and convergent genomic variations that may explain their diversification. Genes associated with aging, survival and homeostasis were enriched in all species. Additionally, positive selection on genes involved in the immune system were disclosed for the two largest species, blue and fin whales. Such genes can potentially be ascribed to their morphological evolution, allowing them to attain greater length and increased cell number. Further evidence is presented about gene regions that might have contributed to the extensive anatomical changes shown by cetaceans, including adaptation to distinct environments and diets. This study contributes to our understanding of the genomic basis of diversification in baleen whales and the molecular changes linked to their adaptive radiation, thereby enhancing our understanding of cetacean evolution.

1. Introduction

One of the greatest challenges in evolutionary biology is explaining the lineage diversification process. It is well known that a combination of intrinsic genetic and extrinsic ecological factors promotes species diversification (Grant & Grant, 2020). However, the relative contribution of these factors remains unclear, particularly when rapid successions of speciation events occur within a lineage. This is particularly problematic in an adaptive radiation, whereby several species rapidly evolve from a common ancestor to occupy different ecological niches (Givnish & Sytsma, 1997; Schluter, 2000). Although several adaptive radiations have been investigated to understand factors promoting rapid diversification (e.g. Beltrán et al., 2002; Freeland & Boag, 1999; Glor, 2010; Smith, Konings, & Kornfield, 2003), the rates and drivers of

speciation events vary considerably among lineages, limiting the understanding of the process of diversification (McGee et al., 2020). The vast majority of adaptive radiation studies have focused on terrestrial or freshwater organisms, with a few notable exceptions from the marine realm (Litsios & Salamin, 2014; Richards & Martin, 2017; Sandoval-Castillo & Beheregaray, 2020). Under the allopatric speciation model, the high species diversity found in the world's oceans has been regarded as a paradox (Bierne et al., 2003) due to the expected high level of connectivity between marine populations. However, the adaptive radiation process provides an alternative explanation to this paradox, emphasizing the role of niche specialization, trophic divergence, and/or ecological adaptation in driving the observed diversity.

Among marine taxa, cetaceans are of great interest for investigating the processes of diversification. Having transitioned from a terrestrial

Abbreviations: MP, mammalian phenotype ontology enrichment; Myr, million years; PSGs, positive selected genes.

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back to an aquatic habitat around 50 million years (Myr) ago, cetaceans underwent radical morphological and molecular changes to adapt to the aquatic environment (McGowen et al., 2020a; Thewissen et al., 2009). They have shown remarkable evolutionary pathways resulting in complex morphological, ecological, and behavioural specializations (Barrett & Schluter, 2008). The diversity of extant cetaceans has been attributed to both allopatric divergence (Steeman et al., 2009) and adaptive radiation (Marx & Uhen, 2010; McGowen, 2011), with evidence of geographic dispersal and ecological divergence (Holt et al., 2020). Some clades, however, exhibit a strong geographical signal, suggesting the possibility of allopatric speciation (Holt et al., 2020). Thus, investigations at a species level that can be extrapolated to higher taxonomic levels might offer valuable insights into the evolutionary pathways among related taxa.

Cetaceans, which encompass whales, dolphins and porpoises, diversified while facing many challenges within their new environment (Nikaido et al., 1999; Steeman et al., 2009; Sun et al., 2019). This diversification resulted in a minimum of 89 currently recognised extant species, divided into two main suborders: 74 belonging to Odontoceti (toothed whales, dolphins and porpoises) and 15 to Mysticeti (baleen whales) (See Fig. 3 of McGowen et al., 2020a; Perrin, 2023). Mysticeti, or baleen whales, possess baleen plates for filter-feeding (Goldbogen et al., 2017), while Odontoceti, or toothed whales, have teeth and are known for their echolocation abilities (Ridgeway & Harrison, 1981). The present study focuses on balaenopterids (the Balaenopteridae family within the Mysticeti), which are represented by nine currently recognised species (Committee on Taxonomy, 2022). These cetaceans are distinguished by their baleen plates and by their ability to communicate with sound signals, engage in social foraging (Tershy, 1992), and undertake migrations spanning vast parts of the globe (Edds-Walton, 1997; Janik, 2014). They are also characterized by a symmetrical skull, paired blowholes, and a lack of ribs that attach to the sternum (Bannister, 2018). Despite similar body shapes, balaenopterids exhibit wide interspecific variation in size. They range from the small common minke whale (*Balaenoptera acutorostrata*), reaching approximately 7–8 m in length, to the largest mammal on Earth, the blue whale (*Balaenoptera musculus*), exceeding 30 m. Recent research suggests that a massive size increase was the last step in the evolution of present-day whales, likely driven by the improved thermal isolation of larger body sizes (Gearty et al., 2018). In addition, baleen whales present a wide range of feeding mechanisms related to a range of energetic strategies and ecological niches (Goldbogen et al., 2017).

Baleen whales are highly mobile and pelagic, so studying them is challenging. Recent advances in conservation genomics have revolutionized the study of non-model species, including cetaceans. The analysis of a species' genome is particularly useful for assessing genetic diversity, population structure, demographic changes, and genetic adaptability in species with global distributions (Carroll et al., 2020; Foote et al., 2019; Jossey et al., 2024). However, little is known about the genomic adaptations that have led to the evolution of baleen whales. The partial mixing of gene pools due to both ancient and recent gene flow between some species of Mysticeti are indicative of the evolutionary complexity of this group (Gatesy & McGowen, 2021). A multi taxa approach is therefore crucial to assess the response of closely related lineages to past natural selection and to understand factors promoting their adaptive radiation.

This study investigates molecular mechanisms of balaenopterid evolution using whole-genome data. The objectives include (1) clarify patterns of genomic diversification in balaenopterids with respect to other cetacean lineages, (2) characterise how the rate of genomic evolution and functional genomic features relate to broad phenotypic diversity, and (3) identify candidate adaptive genomic regions that contributed to the evolution of baleen whales. Baleen whales provide an ideal system to study the genomic underpinnings of diversification in cetaceans and to understand how evolution has shaped genomes and phenotypes during the adaptive radiation of an iconic group of marine

mammals.

2. Methods

2.1. Data acquisition

Genomic data of 17 mammals, including eight mysticetes, seven odontocetes and two terrestrial species (*Hippopotamus amphibius* and *Bos taurus*) were downloaded from NCBI (www.ncbi.nlm.nih.gov), CNGBdb (db.cngb.org) and dnazoo (www.dnazoo.org). *Hippopotamus amphibius*, a semiaquatic species, is the closest relative of extant cetaceans, and *Bos taurus*, with its well-assembled and annotated genome, serves as terrestrial outgroup. Recent phylogenetic studies have shown that the gray whale (*Eschrichtius robustus*) is part of the monophyletic group of the Balaenopteridae family (McGowen et al., 2020a; Themudo et al., 2020; Wolf et al., 2023) and, as such, was included in this phylogenomic analysis. Of the acquired genomes, 15 were assembled at the chromosome level (Table 1).

2.2. Bioinformatics and orthologous genes

All bioinformatics analyses were conducted using the DeepThought HPC (Flinders University, 2021). The BUSCO v5 pipeline was employed for gene prediction and for comparing the mammalian genomes with the Cetartiodactyla orthologous database OrthoDB v10 (Kuznetsov et al., 2023). In BUSCO the MetaEuk (Levy Karin et al., 2020) workflow was used with default parameters and the Cetartiodactyla orthologous to obtain the gene sequences present in each genome. Completeness was assessed based on the proportion of orthologous genes found (Figure S1, initial test for completeness, using 34 whole-genomes available online relevant to the study). Only single-copy genes present in more than 85 % of the species were included for further analyses (Table 1; Figure S2).

From the gene sets identified in over 85 % of the species, multiple nucleotide alignments were created using PRANK with default settings, resulting in 10,159 single-copy genes aligned. As suggested by Jordan & Goldman (2012), PRANK can align sequences reliably even without additional filtering and trimming. Their study suggested that filtering and trimming may sometimes improve phylogenetic signal, but it can also potentially reduce the power for detecting positive selection in certain cases. Similarly, Spielman et al. (2014) noted that no filtering method can improve positive-selection inferences. Thus, PRANK's default settings were used here to maintain robustness without incorporating additional filtering steps. A partitioned file in nexus format with each gene considered a different partition was created using the 'nexusIQ3.pl' in-house script available at <https://github.com/Yuma248/MELFUnction/scripts>.

To infer branch-specific evolutionary acceleration, a phylogenetic tree is essential. By incorporating odontocetes, the semiaquatic and the terrestrial lineages into the alignments, it enabled the transition from conducting pairwise comparisons between balaenopterids and other mysticetes, to inferring common ancestral states and branch-specific changes. These approaches gained strength both statistically and biologically as more genomes of mysticetes were included, facilitating the identification of genes that have undergone accelerated substitution since the most recent common ancestor (Clark et al., 2003). To construct the cetacean orthologous phylogenomic tree, a maximum likelihood approach was employed using IQ-TREE (Minh et al., 2020). The program was run using the phylip file, with the model option `-m TEST`, to determine the best-fit model, which performs model selection (ModelFinder) and tree inference separately for each gene, using the 10,159 aligned genes. *B. taurus* and *H. amphibiosus* were used as outgroups, and the tree was rooted with *B. taurus* and the results were confirmed with 1,000 bootstraps using UFBoot. The final tree was visualized using iTol (Letunic & Bork, 2021).

Table 1
Mammal genomes with over 87% of the genes on the Cetartiodactyla database from OrthoDB. Genomes sourced from NCBI or DNAZoo, along with their respective publication or 'Owner' and year of public availability, where available. The sources include: VGP (<https://vertebrategenomesproject.org/>), NWPU (Northwestern Polytechnology University) and UoA (University of Arizona).

Order	Family	Common Name	Species	Assembly ID	Coverage	No. Scaffolds	Contig N50 (kb)	Publication	Assembly	Busco Coverage
Artiodactyla	Balaenopteridae	Rice's whale	<i>Balaenoptera ricei</i>	Balaenoptera_ricei_HiC	n/a	141,314	71	DNA Zoo	Chromosome-length	C:93.0 %
		Bryde's whale	<i>Balaenoptera edeni</i>	Balaenoptera_bryde	349x	66,600	103,910	Yuan et al. (2021)	Scaffold	C:92.3 %
		Northern blue whale	<i>Balaenoptera musculus</i>	GCF_009873245.2	51.16x	105	6,316	Bukhman et al. (2024)	Reference/Chr-length	C:95.6 %
		North Atlantic fin whale	<i>Balaenoptera physalus</i>	GCA_023338255.1	30x	1,361,899	146	Wolf et al. (2022)	Chromosome-length	C:93.9 %
		North Atlantic humpback whale	<i>Megaptera novaeangliae</i>	GCA_004329385.1	102x	102	12	Tollis et al. (2019)	Chromosome-length	C:94.3 %
		Gray whale	<i>Eschrichtius robustus</i>	Eschrichtius_robustus_HiC	n/a	119,935	67,1	DNA Zoo	Chromosome-length	C:94.0 %
		North Pacific minke whale	<i>Balaenoptera acutorostrata</i>	GCF_000493695.1	92x	10,776	22.69	Yim et al. (2014)	Scaffold	C:95.1 %
		Southern right whale	<i>Eubalaena australis</i>	RWref_HiC	n/a	3,234	27,7	DNA Zoo	Reference/Chr-length	C:94.8 %
		Sperm whale	<i>Physeter macrocephalus</i>	GCF_002837175.2	248x	14,677	42.5	Fan et al. (2019)	Chromosome-length	C:92.2 %
		Gervais' beaked whale	<i>Mesoplodon europaeus</i>	Mesoplodon_europaeus_HiC	n/a	569,347	44.9	DNA Zoo	Chromosome-length	C:87.8 %
		Beluga	<i>Delphinapterus leucas</i>	ASM228892v2_HiC	n/a	6,972	158	DNA Zoo	Chromosome-length	C:95.7 %
		Vaquita	<i>Phocoena sinus</i>	GCF_008692025.1	67.26x	64	20,218	Morin et al. (2021)	Reference/Chr-length	C:95.2 %
		Orca	<i>Orcinus orca</i>	GCF_937001465.1	34x	448	45,583	Foote et al. (2015)	Reference/Chr-length	C:95.7 %
		Commerson's dolphin	<i>Cephalorhynchus commersonii</i>	Cephalorhynchus_commersonii_HiC	n/a	143,722	92	DNA Zoo	Chromosome-length	C:94.1 %
		Bottlenose dolphin	<i>Tursiops truncatus</i>	GCF_011762595.1	63.7x	362	9,739	VGP 2020	Reference/Chr-length	C:95.4 %
Hippopotamidae	Hippopotamus	<i>Hippopotamus amphibius</i>	GCA_023065835.1	69x	697	49,478	NWPU 2022	Chromosome-length	C:95.2 %	
Bovidae	Cow	<i>Bos taurus</i>	GCF_002263795.2	80x	2,210	25,896	UoA 2018	Chromosome-length	C:95.7 %	

2.3. Testing for positively selected genes (PSGs)

Evidence for positive selection on each Balaenopterid species were analysed using the *codeml* package PAML v4.9 (Yang 2007). This package was adapted with an in-house script ‘*paml.pl*’ available at <https://github.com/Yuma248/MELFUnction/scripts>. It is important to note that the true topology within the baleen whales likely remains uncertain. Phylogenomic studies showed direct disagreements (Árnason et al., 2018; McGowen et al., 2020a; Wolf et al., 2023), with some proposing a polytomy for certain branches (Wolf et al., 2023). In particular, high bootstrap support values can be artificially elevated with large datasets, so the assumed topology may still be incorrect. Independent analyses were conducted for each individual species within the mysticetes, the odontocetes and the outgroups. The inclusion of odontocetes and outgroups in the phylogenomic framework was crucial for several reasons. Firstly, a broader context for understanding cetacean evolution was provided, enabling branch-specific evolutionary changes within mysticetes to be assessed more accurately. Secondly, adaptations unique to mysticetes, potentially linked to their aquatic lifestyle and large body size, could be discerned by contrasting PSGs across mysticetes, odontocetes, and terrestrial species.

Additionally, further analyses were performed at key bifurcations within the phylogenomic tree of mysticetes. These bifurcations included the separation between the mysticetes and odontocetes, by using the sperm whale as outgroup, as well as the family Balaenopteridae, which encompasses all balaenopterid species and the gray whale, and the family Balaenidae, represented by the southern right whale. Within the Balaenopteridae family, the bifurcations analysed were those involving the clade containing Bryde’s and Rice’s whales, the clade containing blue, Bryde’s, and Rice’s whales, the clade containing fin and humpback whales, and the clade containing fin, humpback, and gray whales. Additionally, the bifurcation between the monophyletic clade of blue, Bryde’s, and Rice’s whales and the monophyletic clade of fin, humpback, and gray whales was analysed. The analyses were performed at different taxonomic levels to determine if changes in genes are linked to specialized distributions at a family or species level.

The set of 10,159 genes were analysed for signs of positive selection through the application of the branch-site model, which considers the ratio of non-synonymous to synonymous substitutions (dN/dS, represented by ω) to vary across both sites and branches (Yang 2007). The four models used under the branch-site model were M0, M2a1, M2a and M2a_rel. M0 (one ratio) assumes a constant ω ratio across sites and lineages (Goldman & Yang, 1994; Yang & Nielsen, 1998) while M2a1 (nearly neutral) assumes the presence of two classes of sites, one with a fixed ω value between branches and another with a constant ω value across sites (Yang, 2007; Yang & Nielsen, 1998). M2a tests for positive selection by postulating three classes of sites, one with an ω value of 0, one with an ω value of 1, and a third with a non-fixed ω value (Yang, 2007; Yang & Nielsen, 1998). M2a_rel is similar to M2a, with the difference that it employs $\omega > 0$, whereas M2a has $\omega > 1$ (Weadick & Chang, 2012; Yang, 2020). A significance assessment of model fit differences was conducted through a likelihood-ratio test (LRT): model M2a was compared to the neutral and nearly neutral (M0 and M2a1) models, while M2a_rel was compared against the nearly neutral model (M2a1), using an in-house script ‘*lrt.pl*’ available at <https://github.com/Yuma248/MELFUnction/scripts>. Specifically, comparison M2a_rel vs M2a1, have demonstrated notable efficacy in detection of positive selected genes (PSGs) using *codeml* (Álvarez-Carretero et al., 2023). A gene was classified as a ‘candidate gene’ (i.e. gene undergoing positive selection) if it was significant ($\alpha = 0.5$) on all three model comparisons.

The functional implications of the unique genes under positive selection were then analysed using gene identifiers from diverse sources using an in-house script ‘*BUSCO2GKO.pm*’ available at <https://github.com/Yuma248/MELFUnction/>. With this script, each gene had the Gene ID, Gene Ontology (GO) and Kegg Orthology (KO) terms associated

to them. However, due to the extent of genes found in this study these terms are only reported and will not be further explained here.

By using the Gene ID, the genes were then further explored for functional enrichment analysis on the MGI database (Law & Shaw, 2018) through MouseMine (<https://www.mousemine.org/mousemine/begin.do>) (Motenko et al., 2015). Each list of positively selected genes (PSGs), identified for individual species (Table S2) and bifurcations within mysticetes, is evaluated independently (Table S4), and referencing the MGI model organism, *Mus musculus* (common mouse), to identify enriched terms. MGI provides six enrichment widgets, but the Mammalian Phenotype Ontology (MPO) enrichment was chosen because it offers specialized and curated terminology that is specifically tailored to describe phenotypic traits and biological functions relevant to mammalian biology. This enrichment method employed a hypergeometric distribution with the Bonferroni test correction used, with a maximum p-value of 0.05. This approach ensures robust identification of over-represented MPO terms associated with the PSGs, providing insights into the functional implications of gene sets across different species. Enrichment terms specific to each species of balaenopterids and each bifurcation of the balaenopterids, were visualised using RStudio (R Core Team, 2021).

A literature search was used to determine the extent of studies on mysticetes that have previously investigated positively selected genes. This was done using all databases in Web of Science on 23rd February 2023. The literature search was done using: ‘((TS=whale\$ OR TS=baleens OR TS=mysticetes OR TS=mysticity) AND TS=(gene adaptation in whales OR aquatic adaptation) AND (TS=positive adaptation OR TS=PSG OR TS=selection))’, to capture genomic studies, positively selected gene tests and only baleen whales or mysticetes from any journal or article type within the databases.

3. Results

3.1. Phylogenomic tree

A robust dataset of 10,159 single copy and complete genes, focusing on genes present in at least 85 % of lineages across 17 mammalian species, including eight mysticetes, seven odontocetes and two terrestrial species, was assembled. The Odontoceti phylogenomic relationships, in the current study, remains consistent with findings from previous literature (McGowen et al., 2020a), while the Mysticeti phylogeny shows some differences from previous findings. The phylogenomic analysis indicates that most branches among the Mysticeti have strong support with bootstrap values between 95–100 % confidence. The analysis shows that the balaenopterids, including the gray whale (*Eschrichtius robustus*), formed a monophyletic group that is separate from the Balaenidae family, represented here by the southern right whale (*Eubalaena australis*). Bryde’s whales (*B. ricei* and *B. edeni*) and the blue whale (*B. musculus*) formed a well-supported clade. Similarly, but with a lower confidence value of 51 %, the humpback whale (*M. novaeangliae*), fin whale (*B. physalus*), and gray whale (*E. robustus*) form a clade. This lower bootstrap value may be due to several factors, such as insufficient phylogenetic signal in the dataset for this particular grouping or evolutionary rates that vary significantly among the genes sampled. Both clades exhibited a sister relationship to the minke whale (*B. acutorostrata*) (Fig. 1c). These findings support the classification proposed by Wolf et al. (2023) based on 20 kbp fragments from an 1.3 Gbp alignment, which indicated that Rice’s whale (formerly considered a subspecies of Bryde’s whale) is more closely related to blue whales, and that humpback whales, fin whales, and gray whales form a monophyletic group. However, this last grouping is not strongly supported by bootstrapping and, as suggested by Wolf et al. (2023), the placement of the gray whale remains uncertain. The authors proposed a polytomy-like situation for this lineage, where the grouping can vary with sampling of different gene subsets.

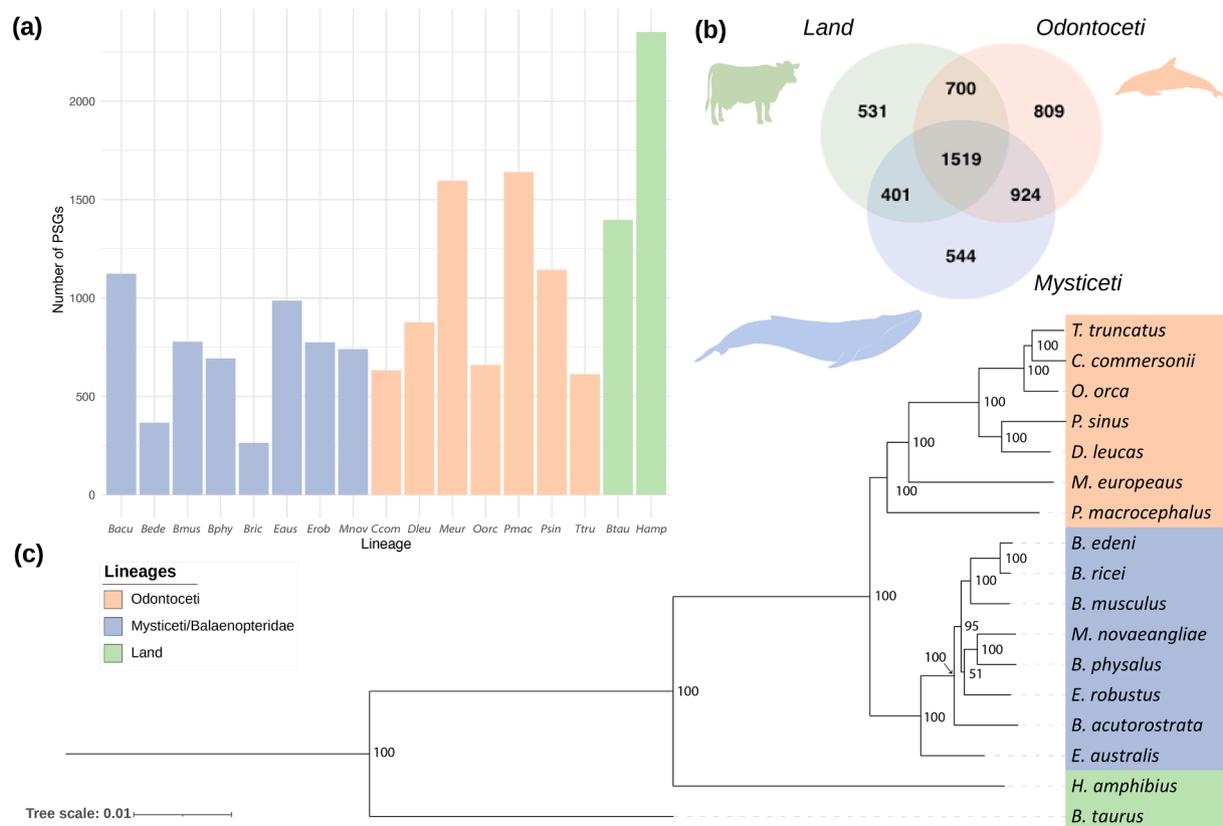


Fig. 1. Comparative Analysis of Candidate PSGs, Lineage-Specific PSG Counts, and Phylogenomic Tree: (a) PSGs – Candidate genes found for each species used in this study. Each lineage is represented by a distinct colour, and the candidate PSGs associated with specific species. (b) Venn diagram of PSGs identified in each group of the species analysed as well as shared among groups, with a total of number of 5,428 PSGs. (c) A phylogenomic multi-species maximum likelihood tree is presented, inferred from 10,159 orthologous genes. Branch lengths represent the genetic divergence between species. The tree depicts the evolutionary relationships among the species under investigation and serves as a framework for interpreting the lineage-specific PSG counts. The numbers on the tree represent the bootstrap values, indicating the support level for each branch. A high-quality version the figure is available on figshare <https://figshare.com/s/a4c63a537aa4563eb8c7>.

3.2. Evidence of positive selection in balaenopterids

To assess positive selection, PAML models were employed, which identified 5,428 genes across all three groups combined (Fig. 1a & 1b; See Figure S3 for the percentage of total genes found to be under selection for each species). Among these, 809 genes were unique to Odontoceti and 531 for terrestrial representatives (see Table S1 for all tested genes for each group). However, given the focus on balaenopterids, the latter results will not be discussed further. Within mysticetes, 3,388 genes exhibited positive selection based on likelihood ratio tests (LRTs) (Fig. 1a). Further analysis focused on 3,150 PSGs unique to balaenopterids, with 2,779 genes showing no duplication across species (Table S2).

The number of PSGs varied among balaenopterids, with notable counts found for the blue whale (*B. musculus*) with 662 genes, followed by the gray whale (*E. robustus*) with 537 genes, humpback whale (*M. novaeangliae*) with 509 genes, minke whale (*B. acutorostrata*) with 508 genes, fin whale (*B. physalus*) with 479 genes, edeni whale (*B. edeni*) with 281 genes, and Rice's whale (*B. ricei*) with 174 genes (Figure S4). Overall, gene functions overlapped between species of balaenopterids, but overlapping genes were less frequent (Figure S5), which may suggest the effects of divergent selection and adaptation to different ecoregions (Sandoval-Castillo et al., 2020) or provide additional evidence supporting the convergent evolution of larger body size (Slater et al., 2017; Wolf et al., 2023) and diving capabilities (Nery et al., 2013; Yuan et al., 2021; Zhu et al., 2018).

Additionally, bifurcation analysis within balaenopterids identified 1,801 PSGs (Table S3), highlighting specific evolutionary adaptations

along the different branches of the Balaenopteridae family (Fig. 2). Distinct sets of PSGs were observed between clades containing blue, Rice's, and edeni whales versus those containing fin, humpback, and gray whales (Table S4).

The literature search disclosed 86 articles, but upon manual filtering, 41 articles discussed gene assessment in cetaceans, and only 14 investigated PSGs in Mysticeti. From the 584 genes reported in these 14 studies (Table S5), only 120 were found in the set of 2,779 unique PSGs for the balaenopterids (Table S2). Therefore, this study presents 2,659 novel PSGs within the balaenopterids.

3.3. Over-enriched terms in the balaenopterids

To understand the functional implications of the positively selected genes, an enrichment analysis was conducted using the MGI dataset through MouseMine. The analysis identified significantly over-enriched MPOs within the set of candidate genes for balaenopterids. This approach employed a hypergeometric distribution and Bonferroni test correction, with a maximum p-value of 0.05, ensuring robust identification of enriched terms.

Based on the MPO enrichment analysis, evidence was found for 81 categories significantly over-enriched in the set of candidate genes for balaenopterids, and 36 categories for the bifurcations within balaenopterids. These mammalian phenotype (MP) categories included mortality/aging (MP:0010768), abnormal survival (MP:0010769), pre-weaning lethality (MP:0010770), homeostasis/metabolism phenotype (MP:0005376), abnormal homeostasis (MP:0001764), abnormal cell physiology (MP:0005621), abnormal blood homeostasis (MP:0009642),

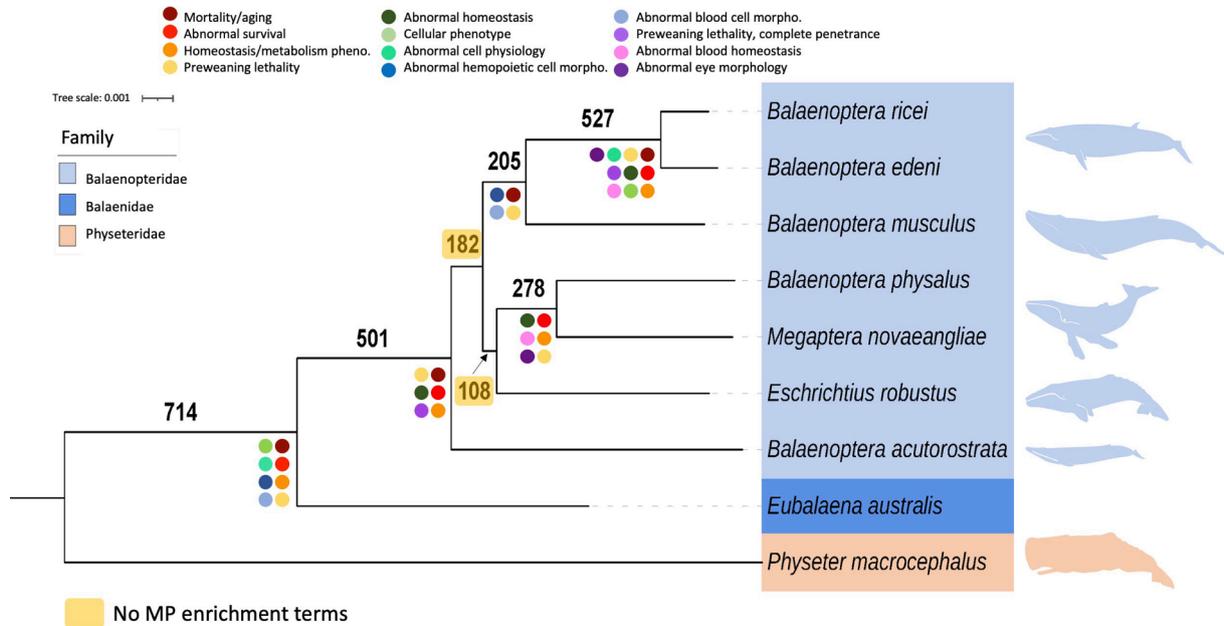


Fig. 2. Positively selected genes during the Mysticeti evolution. Species tree relationships of eight modern cetaceans with complete genome assemblies, estimated from 10,159 orthologs. 1,810 PSGs found specifically for the balaenopterids bifurcations and 714 for the bifurcation with the sister clade balaenids. The tree was rooted using the sperm whale, *Physeter macrocephalus*. Numbers indicate the amount of positively selected genes for each bifurcation in the tree. Circles indicate the top twelve from the thirty-six total enriched MP terms detected on each bifurcation after Bonferroni correction (p-value > 0.05).

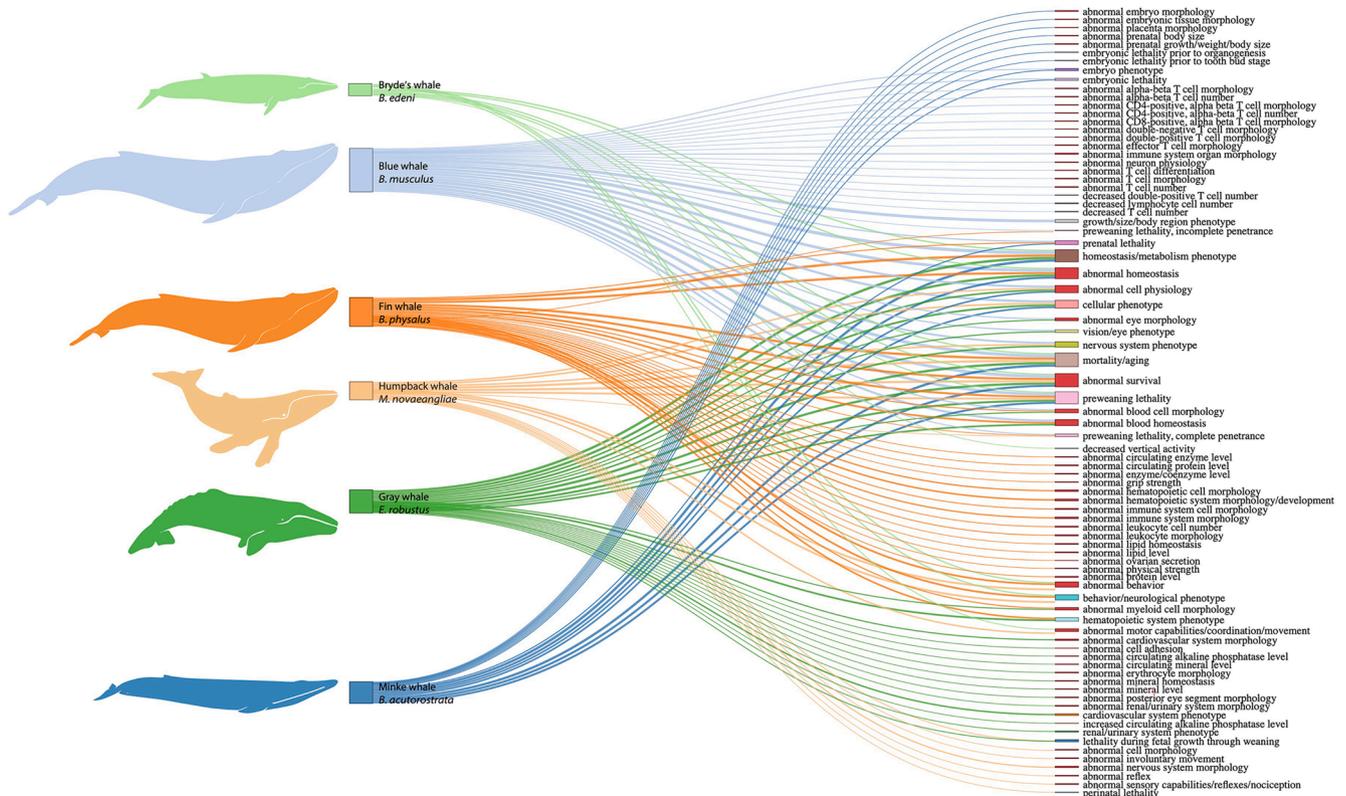


Fig. 3. Sankey diagram illustrating the significant associations between species of balaenopterids and MP Terms, adjusted using Bonferroni correction (p-value ≤ 0.05). The diagram visually represents the flow of significant relationships, with species of balaenopterids on one side and MP Terms on the other, connected by directed flows representing the significant associations. The width of the flows corresponds to the strength of the associations based on the number of terms, highlighting the most significant connections between species and MP Terms in the dataset. A high-quality version of the figure is available on figshare <https://figshare.com/s/a4c63a537aa4563eb8c7>.

among many others (Fig. 3; Table S6 and Figure S6). No enrichment was found for the monophyletic clade including fin, humpback, and gray whales, nor for the bifurcation separating blue, edeni, and Rice's whales, from fin, humpback and gray whales (Fig. 2). Nonetheless, four enrichment terms (mortality/aging, abnormal survival, homeostasis/metabolism phenotype and preweaning lethality) were consistently observed across various bifurcations and were identified during the early stages of balaenopterid evolution (Fig. 2). These findings may indicate adaptive processes across species and different evolutionary timescales of the balaenopterids.

Species-specific terms provided insights into the variations that contributed to the diversification of each species. For example, PSGs were observed in fin whales and blue whales associated with terms on the immune system and anti-cancer mechanisms. And gray whales exhibited positive selection for renal and urinary adaptations, while humpback whales showed positive selection for movement-related terms (Fig. 3; Table S6 and Figure S6).

4. Discussion

The molecular mechanisms of balaenopterid evolution were explored through a comparative approach based on whole-genome data. This was achieved by characterising patterns of genomic diversification in balaenopterids, identifying genomic regions under positive selection in this group, and determining the relationship between genomic evolution and functional genomic features with broad phenotypic diversity.

The evolutionary history of cetaceans and the baleen whale suborder, Mysticeti is characterised by multiple radiation events. These include the transition to an aquatic environment by early cetaceans, the subsequent radiation within Cetacea, and the distinct radiation of baleen filtering within Mysticeti (Steeman et al., 2009; Sun et al., 2013). Recent phylogenetic analyses also suggest further radiations within specific cetacean lineages, such as the rorquals (Árnason et al., 2018; McGowen et al., 2020b). These evolutionary transitions and diversifications have allowed cetaceans to occupy a variety of habitats worldwide, making them a suitable system to study the genomic mechanisms of adaptive radiations. Despite having an axiomatic ecological role, cetaceans face significant threats from anthropogenic factors, which may result in either acute or chronic impacts (Kennish et al., 1984; Kovalenko, 2019; Popper & Hawkins, 2016). As a result of current and emerging threats caused by climate change, and other anthropogenic stressors (Thomas et al., 2016), many balaenopterid populations are struggling. Understanding their evolutionary mechanisms is therefore important to improve the efficiency of management and conservation efforts. Gigantism, diving capabilities, migration, and preweaning lethality provide a focus through which key adaptive strategies enabling the evolution and persistence of balaenopterids can be uncovered. These terms highlight the genetic foundations and phenotypic evolution that enable their ability to manage environmental challenges and reproductive demands.

4.1. Adaptive evolution in balaenopterids

Genes that have undergone positive selection are frequently associated with adaptive phenotypic evolution (Fan et al., 2019) and positive fitness (Chase et al., 2021; Endo et al., 2018; Qiu et al., 2012). The emergence of high-quality genomes from eukaryotes spanning diverse evolutionary lineages has enabled comparative studies of adaptation at the levels of lineages, genes and molecular pathways (Themudo et al., 2020). In spite of technological advances, understanding the diversification processes of the large balaenopterid radiation is challenging (Norris & Hull, 2012). Several studies have investigated PSGs in balaenopterids (Keane et al., 2015; D. Sun et al., 2019; Tollis et al., 2019; Tsagkogeorga et al., 2015; Yim et al., 2014). Some have identified PSGs involved in the immune system, lipid metabolism, and thermoregulation, that may have contributed to the adaptation of balaenopterids to

their aquatic environment (Shen et al., 2012; Tejada-Martinez et al., 2021; Yim et al., 2014). Other PSGs have been related to metabolism and sensory perception (Chikina et al., 2016; Yuan et al., 2021), and likely reflect adaptations to the unique diet and sensory environment of each species. However, previous studies focused on specific genes or utilized smaller gene sets. For instance, Yim et al. (2014) employed a PAML approach similar to ours and remains the only study to date that analysed 9,848 orthologous genes (excluding singletons) for four artiodactyl genomes (minke whale, bottlenose dolphin, cow and pig). Similarly, Yuan et al. (2021) used PAML for 7,525 orthologous genes on 17 marine mammal genomes. In contrast, our study takes a more comprehensive approach, using 17 higher-quality artiodactyl genomes now accessible and analysing a larger orthologous gene set of 10,159. This significantly broadens the scope of identified PSGs and species and enhances our understanding of the potential functions in these species.

In this study, we identified several novel PSGs not previously reported in balaenopterids. For instance, genes such as ACO2, AIFM1, DENND1C and TAT are associated and were found to be enriched for terms like preweaning lethality, mortality/aging, and abnormal survival. Additionally, ABCG8 and ADORA2B are linked to abnormal homeostasis and abnormal blood homeostasis. Other examples include BZW2, associated with decreased circulating iron, cholesterol and glucose levels, which was enriched for the term abnormal homeostasis; and EPO, associated to hypoxia and pericardial edema was related to abnormal homeostasis; among many other genes identified in this study.

These novel findings confirm and expand on the genes previously identified in the literature, such as RAD52, involved in DNA repair; KIF3C, related to intracellular transport; DAGLA, involved in neurotransmitter signalling; NPC1, associated with cholesterol transport (Tsagkogeorga et al., 2015); GOLGA1, associated with Golgi apparatus structure (Yim et al., 2014); and GNPAT, linked to lipid metabolism (Wang et al., 2015). By uncovering these additional PSGs and their associated phenotypic terms, our study provides a broader understanding of the evolutionary adaptations in balaenopterids and highlights the complex interplay of genes involved in their unique adaptations. This comprehensive approach enables a more nuanced comparison and deeper insight into the evolutionary biology of these marine mammals.

4.1.1. Gigantism

Mortality/aging and abnormal survival genes were found to be enriched in all species of balaenopterids and in the branch-set analysis for all baleen whales. The strong selection over these genes could allow the increment in body size without increase of cancer incidence, an adaptation that could explain the Peto's paradox. Peto's paradox refers to the phenomenon where large-bodied, long-lived animals have not shown an increase in cancer rates proportional to their size, in contrast to what might be expected based on the risk associated with accumulating more cells over time (Peto et al., 1975).

Whales have become a subject of significant interest due to their cancer-resistant capabilities, which may be linked to their adaptations to the marine environment (Tejada-Martinez et al., 2021; Tollis et al., 2019; Yim et al., 2014). Recent studies exploring the genome of humpback whales also suggest that the evolution of body mass in cetaceans may be associated with strong selection over cancer resistance pathways (Tollis et al., 2019). As such, cetaceans may be a suitable model for investigating the genetic basis of body size, aging and cancer resistance evolution (Sun et al., 2022), as previously demonstrated for giant terrestrial species (Herrera-Álvarez et al., 2021; Quesada et al., 2019). Additionally, the identification of selection on genes associated to the terms of mortality/aging and abnormal survival may provide insights into how large-bodied whales have managed to evolve without a commensurate increase in cancer incidence, contributing to the understanding of the Peto's paradox.

The much higher rate of body mass evolution in cetaceans compared to their terrestrial relatives has been attributed to factors such as aquatic

weightlessness, abundant diet, thermoregulation, predator defence, and migratory behaviour (Evans et al., 2012; Montgomery et al., 2013). Although baleen filter feeding likely evolved around 25 Myr ago (Peredo et al., 2017; Tsai & Fordyce, 2015), gigantism became ecologically advantageous during the Plio-Pleistocene epoch (around 5.3 million to 2.6 million years ago), likely due to intensified seasonal upwelling (Slater et al., 2017). For instance, the blue whale which can weight up to 180,000 kg (Jefferson et al., 2015), presented in this study 250 genes, such as ATP13A1, ATP18B1, DNAH9, DIS3L2, EHMT1 and FLVCR1, associated to the term growth/size/body region phenotype. Larger body sizes are typically associated to longer lifespans, and genes that exhibit evidence of positive selection were found to play a role in DNA repair, cell cycle regulation, resistance to ageing and cancer confirming what has been previously presented in other studies on cetaceans (Firsanov et al., 2023; Keane et al., 2015). The lack of significant enrichment in the term growth/size/body region phenotype for species other than the blue whale may imply potential constraints stemming from incomplete genome assemblies, quality of annotations, or database limitations. These factors should be considered when interpreting results and assessing the evolution of gigantism in balaenopterids. However, it could also suggest that blue whales, in achieving the largest body mass among balaenopterids, have evolved additional anti-cancer mechanisms facilitating their gigantism.

4.1.2. Diving capabilities and migration

Marine mammals are skilled at deep diving and spend most of their time in deep waters, except when the density of prey is higher near the ocean's surface. Diving capabilities vary among species, with minke, fin, humpback, and gray whales estimated to dive down to 200 m, while the blue, Bryde's, Rice's, and sei whales reach depths exceeding 300 m (Würsig et al., 2018). Their high myoglobin concentration and large muscle mass contribute to the total oxygen stored, particularly in their locomotor muscles (Arregui et al., 2021; Christiansen et al., 2022b). However, deep diving poses a risk of cellular stress, such as hypoxia, oxidative, and osmotic stress (Fabrizius et al., 2016; Ngoh et al., 2011).

Cetaceans have adapted to the hypoxic conditions induced by deep diving, which is crucial for their survival (Tian et al., 2016). Several whale-specific genes have been strongly associated with stress resistance (Yim et al., 2014). For instance, glutathione has been demonstrated to enhance the antioxidant capacity in cetacean cells, protecting against damage caused by reactive oxygen species (ROS) under hypoxic conditions. This protective effect involves genes such as PRDX6, SOD1, and GSTP1 (Yim et al., 2014). This study identified enriched terms in balaenopterids associated with either the homeostasis/metabolism phenotype or blood homeostasis. Homeostasis phenotype refers to the set of traits, functions, and mechanisms that enable an organism to maintain stable internal conditions despite changes in the external environment (Weinstein, 2000). It involves the regulation of various physiological and metabolic processes, such as temperature, fluid balance, pH, and nutrient levels, to keep them within a narrow range suitable for optimal functioning (Weinstein, 2000).

Enriched homeostasis/metabolism terms were consistently observed in different species (blue, edeni, fin and gray whales) and persist across various bifurcations in the evolutionary lineage of balaenopterids, highlighting their adaptive strategies in challenging environments facilitated by their large body size and robust homeostatic mechanisms. Some species of balaenopterids exhibit a dynamic balance between short-term residency in cold, productive areas at low latitudes, and lipid storage (Brodie, 1975, 1977). Together with their increment in size, the migratory response to seasonal changes and cold resistance to guarantee an increment of their lipid storage may be partially attributed to how homeostasis is maintained as a response to extreme environments.

Previous studies compared deep-diving odontocetes, such as sperm whales and Blainville's beaked whales, which dive over 1,000 m, focused on hypoxia related genes, and identified genes under selection related to myoglobin (Nery et al., 2013), in genes like for example SETX,

GIF, and TMPRSS11D (Yuan et al., 2021). While baleen whales do not exhibit PSGs specifically related to hypoxia, they may have evolved alternative adaptations to compensate for reduced diving capabilities. For instance, fin and gray whales, exhibited enrichment in myeloid cell morphology and the hematopoietic system phenotype, indicating adaptations that supports the immune response under high pressure conditions during dives. For example, the genes found to be related to the term hematopoiesis (i.e. CD55, ERG and FLNB), which occurs in the bone marrow and involves stem cell differentiation into blood cell types responsible for oxygen transport, immune response, and haemostasis (Itkin et al., 2016), can further highlight the adaptations for coping with the pressures at greater depths during diving.

In the current studies, no signal of positive selection was found for previously reported genes related to cetacean diving capabilities. However, this study has a broader approach and evidence was found for PSGs enriched on the baseline of the balaenopterids branch, such as those related to abnormal blood cell morphology, abnormal apoptosis (i.e., the physiological process of cell death that occurs during an organism's growth and development) and abnormal erythrocyte morphology (i.e., the primary function involved in the transportation of oxygen and carbon dioxide to and from the body's tissues). These adaptations may be linked to the abilities of whales to dive and grow larger while protecting themselves against tumour development (Tejada-Martinez et al., 2021). Although baleen whales do not possess the extensive diving skills of odontocetes, they have developed mechanisms to cope with the pressure in the marine environment, showing additional evidence of convergent evolution between cetaceans.

4.1.3. Prewearing lethality

Prewearing lethality was another term consistently found within the different species of balaenopterids and prevailed in the bifurcations of the different branches. Here, baleen whales exhibited signals of positive selection over multiple genes associated with the MP term preweaning lethality. The presence of such signals suggests an evolutionary advantage associated with managing the substantial risk of mortality from fertilization to weaning, usually occurring at 5–6 months of age for calves (Chivers, 2009). This adaptation may reflect strategies honed through natural selection, where genes associated with preweaning lethality undergo positive selection to enhance the survival prospects of offspring. By favouring traits that reduce mortality rates during the vulnerable preweaning period, balaenopterids can increase the likelihood of reproductive success and passing on advantageous genetic variations.

Baleen whale females generally outweigh males because of potential energy demands of reproduction and increased fat storage (Ralls, 1976). However, the cost of foetal growth increases rapidly during gestation, particularly in the latter stages of pregnancy, as foetal mass increases exponentially with foetal length (Christiansen et al., 2022a,b). A similar phenomenon was observed in southern elephant seals, where pups experienced a remarkable average growth rate during the lactation period, with the proportion of body mass represented by fat increasing from less than 3 % at birth to 12.7 % at weaning (Hindell et al., 1994). These findings highlight the significance of maternal energy reserves and body condition in shaping the growth rates and overall health of offspring in different marine mammal species.

Estimating neonatal mortality in baleen whales is challenging due to limited data. Growth models revealed that baleen whale calves can nearly double their length and achieve nearly eight times their body mass during the nursing period (Agbayani et al., 2020; Fortune et al., 2021). Nevertheless, precocial development in mammals has detrimental implications on the body development of the infant (Ashmeade et al., 2007; Gaillard et al., 1997; Lezama-García et al., 2022; Magrini et al., 2023). Mothers under poor body conditions can be a significant risk for the survival of their calves. In humpback whales, the Gulf of Maine population showed a one-year birth interval rate of 6.3 % (Clapham et al., 1993), while a higher rate of 14 % was reported from

the Hawaiian breeding range (Glockner-Ferrari & Ferrari, 1990). Clapham et al. (1993) partially attributed this to neonatal mortality before the mother's return to high-latitude feeding grounds, as any calf that dies in the West Indies or during migration north would not be recorded in the Gulf of Maine later in the year. Considering the substantial lactation cost in baleen whales (Lockyer, 1976, 1981), females with shorter interbirth intervals, notably one year, and with poor body condition (Christiansen et al., 2014), could experience higher calf mortality. It is important to consider the vulnerability of pregnant females, embryos, and calves during this period. Anthropogenic threats and disturbances to the mother and calf, such as navigation, seismic activities and other offshore developments, can have a significant impact on whale populations (Pirotta et al., 2018). Previous studies have shown that reduced survival of embryos and calves plays an important factor for the recovery of protected populations (Cramer et al., 2008; Hin et al., 2019), underscoring the necessity of considering these factors in the management and conservation of baleen whale populations, with special attention to their breeding and calving areas.

4.2. Species-specific adaptations

4.2.1. Immune system in the largest species, the blue and fin whales

Despite the common adaptations found in balaenopterids, species-specific adaptations have likely evolved independently, contributing to their success during diversification. For instance, blue and fin whales were found to exhibit unique immune system adaptations that may play a crucial role in their survival. In this study, positive selection was observed for genes associated with terms enriched with immune responses, particularly within T cells for blue whales and leukocyte cells for fin whales.

In blue whales, there is significant enrichment in genes related to the terms of morphology and number of cytotoxic T cells (CD8 +) and CD4 + cells, as well as alpha-beta T cell differentiation. These T cells are vital for immune responses, including tumour recognition and fighting against cancer-infected cells and foreign antigens (Van den Eynde & van der Bruggen, 1997). CD4 + and CD8 + are responsible for identifying and fighting against cancer-infected cells and foreign antigens (Smith-Garvin et al., 2009). The presence of tumour-infiltrating lymphocytes, particularly CD8 + T cells, is a favourable prognostic marker across various solid tumours (Fridman et al., 2012). However, these cells often struggle to eliminate cancer cells due to immunosuppressive mechanisms within the tumour microenvironment (Chen & Mellman, 2013; Schreiber et al., 2011). Optimizing CD4 + T cell assistance has shown promise in enhancing cancer immunotherapy outcomes in humans (Borst et al., 2018). The significant enrichment of genes related to morphology and the number of cytotoxic T cells (CD8 +) and CD4 + cells, along with alpha-beta T cell differentiation in blue whales, underscores their evolutionary advantage in combating cancer as they acquire a long body size and maintaining immune resilience.

In fin whales, there is clear evidence of immune system enrichment, particularly in leukocyte cells, which suggests a robust immune system crucial for their adaptation. The emphasis on leukocyte cells suggests a specialized immune response tailored to combat environmental pathogens and maintain overall health in fin whales. These specialized immune responses, critical for tumor recognition, defense against foreign antigens, and enhancing resilience in dynamic marine ecosystems, highlight adaptations that contribute to the blue and fin whales thriving despite environmental and physiological challenges in their marine habitat.

4.2.2. Feeding and diving strategies of gray whales

While most of the water needed by whales is obtained through metabolising fat from their food, they may still consume seawater in certain situations (Ortiz, 2001). Gray whales use a unique benthic-suction feeding mechanism (Werth et al., 2016). Their kidneys have specialized adaptations, such as glycogen stores, vasa recta bundles, and

a sporta perimedullaris musculosa, to support diving and produce concentrated urine in hyperosmotic environments (Pfeiffer, 1997; Vardy & Bryden, 1981). Despite these adaptations, their kidney efficiency is lower than expected, but they compensate with hormonal regulation (Bester, 1975; Vardy & Bryden, 1981). The analysis of gray whales revealed a high enrichment of genes associated with renal/urinary and mineral homeostasis, as well as their cardiovascular system and circulating alkaline phosphatase and minerals. These genes are thought to be critical in maintaining blood pressure and water balance, especially in environments with elevated sodium intake and exposure to sediment-laden conditions on the seafloor (Oliver & Slattery, 1985). These adaptations are probably allowing gray whales to efficiently process high-salt intake and to extract maximum nutrients from their food source, but it is also placing them at a higher risk to contaminants accumulated on the seabed (Dehn et al., 2006).

4.2.3. Movement and reflexes of humpback whales

Despite the common adaptations found in balaenopterids, adaptations within each species have likely occurred independently and contributed to their success during diversification. For instance, humpback whales, are renowned for their agility despite their large (12–16 m) and rigid bodies (Clapham, 2018). They exhibited positive selection in genes associated with reflexes, sensory capabilities, coordination, movement, and involuntary movements. These adaptations might relate to their specialized foraging techniques and famous singing repertoire, aiding their communication and mating system (Clapham, 2000). Humpback whales exhibit unique behaviours among cetacean species, as they are the only ones known to form leks. Humpback males engage in vigorous combat for access to females (Clapham, 2000). The presence of protuberances along the leading edge of its pectoral flippers have been suggested as the reason for their agility. Fish and Battle (1995) hypothesized that these structures play a role in the species' feeding strategy. However, it could also be attributed to their significant male-male competition (Chu & Nieukirk, 1988; Tyack & Whitehead, 1982). Further studies noted that their tight turning radii during extreme manoeuvres, such as bubble netting (Baker & Herman, 1984; Tyack & Whitehead, 1982), are unique, and the protuberances may facilitate passive flow control and/or drag reduction (Fish & Lauder, 2006). Positive selection was observed in genes enriched within the humpback whale species associated with reflexes, sensory capabilities, coordination, movement, and involuntary movements. Given their unique feeding techniques and famous singing repertoire, it is possible that these whales also possess gene variations that contribute to their specialized adaptations for foraging, male competition and communication.

5. Conclusions

This study provides further evidence of the success of balaenopterids and they underwent adaptive evolution, enabling them to thrive in their aquatic habitat and diversify into distinct species with variations in size, morphology, mating systems, feeding strategies and diving abilities. By identifying positively selected genes and enriched mammalian phenotypic terms, this research highlights the genetic and physiological adaptations that underpin the diversification and specialisation of balaenopterids. The work advances our understanding of the genetic mechanisms behind the evolution of baleen whales, offering new insights into their evolutionary triumph.

CRedit authorship contribution statement

Gabrielle Genty: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jonathan Sandoval-Castillo:** Writing – review & editing, Validation, Software, Methodology, Data curation. **Luciano B. Beheregaray:** Writing – review & editing, Validation, Supervision. **Luciana M.**

Möller: Writing – review & editing, Validation, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

I have share the link for the online genomes on Table 1 and the supporting tables are available from Zenodo: 10.5281/zenodo.10703298

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.gene.2024.148822>.

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