Novel insights on aquatic mammal MHC evolution: Evidence from manatee DQB diversity

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A R T I C L E   I N F O

A B S T R A C T

The low diversity in marine mammal major histocompatibility complex (MHC) appears to support the hypothesis of reduced pathogen selective pressure in aquatic systems compared to terrestrial environments. However, the lack of characterization of the aquatic and evolutionarily distant Sirenia precludes drawing more generalized conclusions. Therefore, we aimed to characterize the MHC *DQB* diversity of two manatee species and compare it with those reported for marine mammals. Our results identified 12 and 6 alleles in *T. inunguis* and *T. manatus*, respectively. Alleles show high rates of nonsynonymous substitutions, suggesting loci are evolving under positive selection. Among aquatic mammals, Pinnipeda *DQB* had smaller numbers of alleles, higher synonymous substitution rate, and a dN/dS ratio closer to 1, suggesting it may be evolving under more relaxed selection compared to fully aquatic mammals. This contradicts one of the predictions of the hypothesis that aquatic environments impose reduced pathogen pressure to mammalian immune system. These results suggest that the unique evolutionary trajectories of mammalian MHC may impose challenges in drawing ecoevolutionary conclusions from comparisons across distant vertebrate lineages.

1. Introduction

Marine mammal immunogenetics has been the focus of growing research to assist with characterizing genetic diversity in imperiled populations and better understand the evolution and physiology of adaptive immunity in a radically different environment compared to model terrestrial mammals. Major histocompatibility complex (MHC) genes have key functions in the adaptive immune response by encoding polymorphic antigen presenting proteins. Research in marine mammals MHC genes began the late 1980s assessing diversity on two major

taxonomic groups, Cetacea and Pinnipeda (Acevedo-Whitehouse et al., 2018; Manlik, 2015; Moreno-Santillán et al., 2016; Murray and White, 1998; Slade, 1992; Trowsdale et al., 1989; Weber et al., 2004; Xu et al., 2010).

The first reports on Cetacea (Trowsdale et al., 1989) and Pinnipeda (Slade, 1992) MHC found restricted polymorphism. This observation led to the hypothesis that the marine environment may challenge populations with reduced selective pressure on the MHC, leading to relaxed evolution and smaller gene diversity. Slade (1992) also provided an important framework for future research on the topic, suggesting three predictions to be tested: i) low MHC diversity will be found in all marine mammals; ii) completely marine Cetacea and Sirenia will have lower MHC diversity compared to Pinnipeda; and iii) higher MHC diversity will be found in Pinnipeda species that spend more time ashore and in seal colonies close to terrestrial animal populations. Since then, several reports, with species under varied conservation status, have provided mixed support to this hypothesis (refences listed in Supplementary Material 1). Most of these studies focused on Slade's (1992) first prediction, including an elegant comparison of cetacean and terrestrial mammal *DQB* genes, suggesting indeed weaker balancing selection in the former (Villanueva-Noriega et al., 2013). Overall, *DQB* diversity has been more comprehensively characterized across several species of marine mammals, with less reports on other MHC genes, such as *DRB, DQA* and class I loci.

Despite the overall advance in understanding aquatic mammal MHC evolution, the lack of reports on sirenians hampers a more comprehensive analysis. The order Sirenia is currently represented by two extant families and genera, including three manatee species and one dugong. While dugongs are the only exclusively marine sirenian species, manatees can be found either obligately in freshwater (the Amazonian manatee *Trichechus inunguis*) or in coastal marine and freshwater environments (the West Indian manatee *T. manatus* and African manatee *T. senegalensis*, with wide variation in usage of both habitats by distinct populations (Castelblanco-Martínez et al., 2021)). The scarcity of fossil records and the availability of a single manatee species genome poses an obstacle on the understanding of evolutionary relationship among the three taxa. Nonetheless, the most comprehensive analysis to date, using a mitogenome from each manatee species, supports the divergence of *T. manatus* and *T. inunguis* around 1.3 mya, with *T. senegalensis* as a sister taxon, sharing a common ancestor with the other species 6.5 mya (de Souza et al., 2021).

Presently the three recognized manatee species are all vulnerable to extinction due to habitat loss, hunting and other anthropogenic factors (IUCN (Deutsch et al., 2008; Keith Diagne, 2015; Marmontel et al., 2016);). Reports on West Indian manatee populations showed relatively low genetic diversity in mtDNA markers in most of their range and population fragmentation, with three lineage clusters of low connectivity (Vianna et al., 2006). The Brazilian population of *T. manatus* has low mtDNA haplotype diversity (Luna et al., 2012; Vianna et al., 2006) and microsatellite diversity (Luna et al., 2021; Luna, 2013). Despite current trends of population reduction, the Amazonian manatee bears greater genetic diversity and lower population structure across its distribution (Luna et al., 2021; Satizábal et al., 2012; Vianna et al., 2006). Despite separation of these species around 1.3 million years ago (de Souza et al., 2021), hybridization has been reported in their sympatric region (Garcia-Rodriguez et al., 1998; Vianna et al., 2006), however its degree in current populations is under discussion (Luna et al., 2021; Vilaça et al., 2019). Overall, functional genetic diversity has been poorly investigated and this may be the next step to further understand the adaptive potential and best maintain both species.

To our knowledge there is a single report on manatee MHC (Vela and González, 1999), however we could not find any peer-reviewed publications with such data. Information on the variability of class II MHC genes such as the *DQB* locus can also provide further knowledge on the vulnerability of the manatee populations and serve as a tool for decision-making during animal release in the wild. This paper aims to characterize *DQB* polymorphism in two species of manatees and provide the first attempt to address Slade's (1992) second prediction by comparing the diversity of two fully aquatic mammalian clades (Sirenia and Cetacea) to Pinnipeda, compiling the largest MHC sequence dataset from marine and aquatic mammals to date.

2. Methods

2.1. Sirenia DQB diversity characterization

2.1.1. Sample collection and DNA extraction

Samples were obtained from 24 *T. inunguis* from Brazil, Pará State, and 40 *T. manatus,* including individuals from Brazil (n = 26), Belize (n = 4) and Florida (n = 10) (Fig. 1). Blood samples were preserved in EDTA solution and frozen until DNA extraction. DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen), following the manufacturer's protocol. All procedures were approved by ethics committee and samples were exported under CITES permit (see Ethics Statement).

2.1.2. DQB genotyping and analysis

Primers were designed using NCBI/Primer-Blast to amplify the second exon of the *DQB* gene from the West Indian manatee genome assembly (Gene ID: 101345015). The designed primer pair TmaDQBF 5′-TCACCGCAGAGGATTTCGTG-3′ and TmaDQBR 5′-CGACTCCTGGA-GACTCAACC-3′ anneals in the beginning of exon 2 and inside intron 2, resulting in a 344 bp amplicon. Primer-Blast using this primer set results in three hits in the African elephant genome, all three predicted *DQB* genes or pseudogenes. Theoretical PCR products in the elephant genome range from 341 to 344 bp and primers have only one to three mismatches to those *DQB* sequences, suggesting the primer set indeed locate to conserved portions of *DQB* from Tethytheria (clade including Sirenia and Proboscidea). Due to lack of *T. inunguis* genomic resources, the amplification of *DQB* in this species was determined experimentally. The characterization of more alleles in *T. inunguis* compared to *T. manatus* (see below in results) suggests the interspecific primers used are indeed able to characterize *DQB* diversity in both species. *DQB* was amplified using GoTaq PCR Kit (Promega), following manufacturer's protocol, using 0.1 μM of each primer. DNA concentration was optimized individually for each sample. The cycling protocol consisted of initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturing at 94 °C for 30s, annealing at 60 °C for 30 s and extension at 72 °C for 40 s,
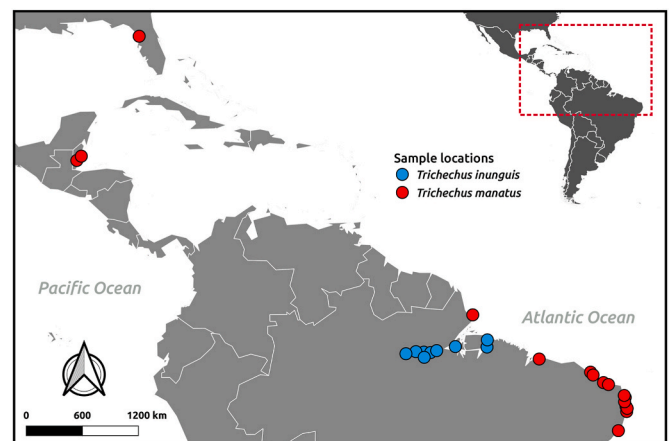

Fig. 1. Map of locations where manatees were sampled in this study. Positions are marked from the reference locality animals were rescued, since samples were not georeferenced during collection. Only samples with known locality (e. g., beach, river or city) are shown; for some samples, only the State from which the sample was collected was annotated, and therefore are not shown in this map. Sample locations are noted for both manatee species and may represent one or more samples.

followed by a final extension at 72 °C for 2 min. *DQB* products were confirmed in a 1% agarose gel. Samples were genotyped by a combination of sequencing cloned PCR products, next generation sequencing (NGS) and phasing diploid genotypes. Sequences were considered true alleles if scored by at least two of the three aforementioned methods, occurring in more than one sample, and compatible to the sample's diploid Sanger phase (i.e. all polymorphic peaks in the sample's Sanger sequence are explained by the combination of the genotyped alleles). Detailed methods for the sequencing and genotyping are provided in the Supplementary Material 2.

Polymorphism summary statistics were assessed in DNAsp v.6. To assess evidence of selection, the rates of synonymous and non-synonymous substitutions were calculated (dN/dS) for the entire exon 2, the peptide binding sites (PBS, defined by (Brown et al., 1993) and (Fremont et al., 1998)), and the non-PBS positions, using the Li-Wu-Luo method, implemented in the software MEGA X (Kumar et al., 2018). Z-test was used to test for the significance of dN/dS ratios, also in MEGA X.

For the site-by-site selection test, we used several methods implemented within the Datamonkey server (i.e. SLAC, FUBAR, MEME and FEL). Because recombination may lead to false positive results in likelihood-ratio tests (Anisimova et al., 2003), evidence for recombination between *DQB* alleles was assessed using the GARD algorithm from the Datamonkey website (Weaver et al., 2018), and selection tests were performed using all *Trichechus* alleles as one (no recombination) or three partitions (based on recombination breakpoints evidenced by GARD). Given that MHC alleles undergo recombination and gene conversion-like events, we used 1000 bootstrap iterations in Splitstree (Huson and Bryant, 2006) to better represent the evolutionary relationship between sequences.

The p-distance between allele sequences from each genotyped individual were calculated in MEGA X and compared with the p-distance expected from all allele pairwise combinations (i.e. all possible genotypes given the alleles found in each species; since the sample's genotype would be a subset of all pairwise combinations, no statistic test was performed.

2.2. Revisiting Slade's (1992) second prediction

We searched scientific repositories to compile an extensive list of class II diversity reports including many Cetacea and Pinnipeda species. The full list of consulted papers is given in Supplementary Material 1. In this analysis, we included all reports on *DQB* diversity that deposited sequences in public repositories. Because of the variety of reported measures of diversity and therefore no uniformity across reports, we compared reported number of alleles and used sequences to calculate molecular diversity across the three aquatic mammal clades, defined as Cetacea, Pinnipeda and Sirenia. For allele number, only reports using sample sizes greater than ten were analyzed. Correlation between sample size and number of alleles was assessed by Spearman correlation in the whole dataset and inside each clade. Differences in allele number between mammals from different habitats was assessed by Wilcoxon-Mann-Whitney test (comparisons were made between exclusively marine mammals and aquatic mammals that inhabits freshwater, exclusively or not).

Sequences were downloaded to create datasets of allelic variants among clades and subclades (i.e., Mysticeti, Odontoceti, Phocidae, Otariidae, Odobenidae and Trichechidae). Because of varying amplification methods, sequences were of different sizes, sometimes covering distinct portions of the *DQB* exon 2. Most Cetacea reports use Tsuji et al. (1992) primers to amplify 172 bp of the *DQB* exon 2 (see Murray et al., 1995); some reports of Pinnipeda also use the same amplification methods, but others use distinct primer pairs. Since the size (and hence the number of predicted PBS) of the sequence may impact the molecular diversity, we analyzed sequences encompassing two regions: 1) the whole exon 2 region, including all alleles irrespective of size and 2) 172

bp of the exon 2, trimming all longer sequences and excluding sequences smaller than 172 bp.

Identical sequences inside each dataset were removed using Fasta Tools Unique Sequences (in https://www.ncbi.nlm.nih.gov/CBBresearch/Spouge/html_ncbi/html/fasta/uniqueseq.cgi) and double checked by pairwise distance computation in MEGA version X (Kumar et al., 2018); alleles with "0" distance but non-overlapping sequences were kept. Using MEGA X, we calculated pairwise dS and dN distances and amino acid p-distances across alleles. dN/dS ratio was calculated as described above, removing "not computed (n/c)" values from the analysis. Amino acid distances were compared using Kruskall-Wallis rank sum test, using pairwise Wilcoxon rank sum test to check differences in protein diversity among clades.

Statistical tests and graphs were made in RStudio; scripts and datasets are provided on Figshare (Sá et al., 2021). Figures were edited in Adobe Photoshop v22.1. To test whether Pinnipeda *DQB* evolved under intensified/relaxed selection compared to other aquatic mammals, we used RELAX (Wertheim et al., 2015), implemented in Hyphy (Kosakovsky Pond et al., 2020), using Pinnipeda *DQB* as the test dataset and other aquatic mammals *DQB* as reference dataset. Results from the 172 bp dataset did not change the main conclusions of this study, therefore only the results from the more comprehensive dataset were reported.

3. Results

3.1. Sirenia DQB diversity

A total of 12 alleles were identified in *T. inunguis* and 6 alleles in *T. manatus*. Four alleles where shared between both species, resulting 14 uniquely identified Sirenian alleles. There was no evidence of deletions, insertions or stop codons in the analyzed sequences (Fig. 2A). Most manatee samples possessed one or two distinct alleles, whereas only one *T. inunguis* sample had evidence of amplification of two additional alleles for a total of four *DQB* alleles. All sequences scored high similarity to the *DQB* sequence from the *T. manatus* genome (GCF_000243295.1), although no allele was identical to the genome *DQB* allele (which may be an artifact of assembly, Gene ID: 101345015). The amplification assay resulted in a fragment of 304 bp (without primers), including 259 bp of the *DQB* exon 2 (86 codons). In the intron 2 sequence, we were able to find two SNPs (alignment position #289 G > C and #301 T > G).

Polymorphism statistics are summarized in Table 1. Overall, *T. inunguis* had higher genetic diversity compared to *T. manatus*, including greater number of alleles, haplotype, and nucleotide diversities (Table 1). Thirty-four of 259 (13,12%) exon 2 nucleotides were variable among all manatee alleles, whereas 21 of 86 (24,41%) amino acid sites varied among alleles. Considering the position in the protein, 14 of 22 (63,63%) peptide biding sites (PBS) position, according to Fremont et al., (1998), and 13 of 23 PBS (56,52%) positions, according to Brown et al. (1993), varied among all manatee alleles. Mutations on the exon 2 of manatee *DQB* largely resulted in amino acid substitutions. Ratios of dN/dS showed evidence that the *DQB* loci is evolving under positive selective pressure, particularly in the PBS (Table 2).

Different selection algorithms revealed evidence for distinct sites under positive selection (Table S1, supplementary material 3). All sites subjected to positive/diversifying selection were PBS, and the residues with overall agreement of multiple positive selection tests were 11, 13, 26, 71 and 74 (Table S1). The only residue with evidence of negative selection was the position 12 residue (Table S1).

Allele frequencies are summarized in Table S2 (Supplementary material 3). The allele with the highest frequency in *T. inunguis* was *TrinDQB*01* and *08*, other alleles varied from 4% to 14%. Overall, *T. manatus* had four alleles with high frequency, however their frequency varied between populations: alleles *TrmaDQB*01*, *05* and *06* were absent in animals from Belize and Florida; allele *TrmaDQB*04* was absent in Brazilian animals.

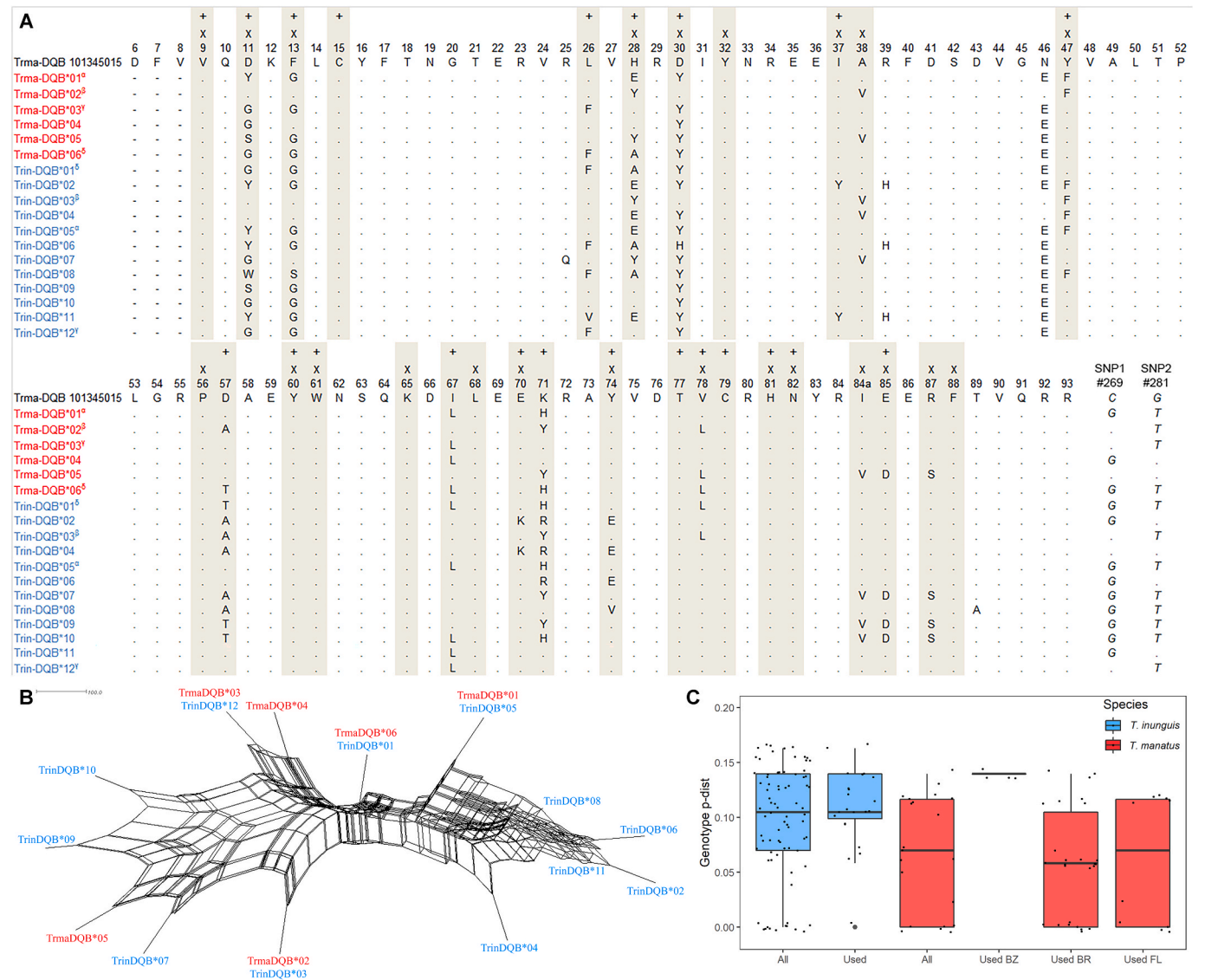*DQB* allele phylogenetic networks revealed trans-species

Fig. 2. *DQB* allele diversity in manatees. **A)** Alignment of *DQB* exon 2 predicted protein sequence. Dots represents identity with the first sequence. Above the alignment, "+" indicates peptide binding sites (PBS), according to Fremont et al. (1998); "x" indicates PBS, according to Brown et al. (1993); number refer to the residues of the *DQB* molecule. **B)** Phylogenetic network showing the relationship of manatee *DQB* exon 2 alleles. **C)** Distribution of amino acid p-distance between alleles in manatee genotypes. "All" refers to the p-distance between all possible pairwise combinations from the alleles in each species, including homozygotes. "Used" refers to the actual combination of alleles in each sampled population. BZ, Belize; BR, Brazil; FL, Florida. Trma, *Trichechus manatus* (in red); Trin, *T. inunguis* (in blue).

Table 1
Polymorphism statistics in two species of manatee, including subpopulations.

Population	N	Size (bp)	Na	S	Hd ±SD	π	k	θw*±SD
Trichechus sp.	64	259	14	34	0.874± 0.014	0.04110	10.644	0.2413 ± 0.00414
T. inunguis	24	259	12	34	0.912± 0.016	0.04968	12.868	0.02931 ± 0.00503
T. manatus	40	259	6	23	0.762 ± 0.023	0.03149	8.155	0.01793 ± 0.00374
Florida	10	259	3	16	0.542 ± 0.076	0.02374	6.147	0.01741 ± 0.00435
Belize	4	259	2	16	0.571 ± 0.094	0.03530	9.143	0.02383 ± 0.00596
Brazil	26	259	5	23	0.655 ± 0.041	0.02720	7.044	0.01965 ± 0.00410

N, number of samples; S, Polymorphic (segregating) sites; Na, number of alleles; Hd, Haplotype diversity; π, nucleotide diversity; k, average number of nucleotide differences; θw, Theta (per site) from S ± standard deviation of theta (free recombination).

polymorphisms between both manatee species, including four shared alleles (Fig. 2B). The relationship between alleles suggests the occurrence of recombination and/or gene conversion-like events, also

suggested by the GARD algorithm (Table S1). Two clusters were formed in the network, the first comprised by alleles *TrinDQB*03, *07, *09, *10, *12* and *TrmaDQB*02, *03,*04, *05,* while the other cluster comprised

Table 2
Rates of non-synonymous to synonymous substitutions (dN/dS) and nucleotide divergence for the second exon of MHC *DQB* locus in aquatic mammals.

Clade	Na	Total (267 bp)			PBS (90 bp)			Non-PBS (177 pb)		
		dN ± SE	dS ± SE	dN/dS	dN ± SE	dS ± SE	dN/dS	dN ± SE	dS ± SE	dN/dS
Sirenia *DQB*	14	0.069 ± 0.017	0.017 ± 0.012	4.05*	0.186 ± 0.048	0.032 ± 0.030	5.81*	0.009 ± 0.006	0.013 ± 0.014	0.69
TrmaDQB	6	0.056 ± 0.016	0.021 ± 0.016	2.66*	0.152 ± 0.044	0.040 ± 0.043	3.80*	0.005 ± 0.006	0.016 ± 0.016	0.31
TrinDQB	12	0.071 ± 0.018	0.016 ± 0.011	4.43*	0.190 ± 0.049	0.027 ± 0.022	7.03*	0.011 ± 0.006	0.013 ± 0.013	0.84
Cetacea *DQB*	170	0.101 ± 0.023	0.046 ± 0.016	2.19*	0.286 ± 0.076	0.107 ± 0.061	2.67*	0.029 ± 0.010	0.037 ± 0.017	0.78
Pinnipeda *DQB*	38	0.063 ± 0.020	0.058 ± 0.018	1.08	0.203 ± 0.074	0.173 ± 0.065	1.17	0.018 ± 0.005	0.035 ± 0.012	0.51

TrmaDQB, *Trichechus manatus*; TrinDQB, *T. inunguis*; Na, number of alleles; SE, standard error; PBS according to Brown et al. (1993) and Fremont et al. (1998); *Z-test $P < 0,05$.

of the sequences *TrinDQB**02, *04, *05, *06, *08, *11*, and *Trma*DQB*01* (Fig. 2B); the alleles *TrinDQB*01* and *TrmaDQB*06* lied in between both clusters. Nonetheless, the clusters were highly related, with little distance.

Amazonian manatee genotypes were enriched in the upper quartiles of the amino acid p-distance between all possible combination of alleles (Fig. 2C); for this comparison, the *T. inunguis* sample with four alleles was removed. On the other hand, *T. manatus* genotype p-distance distribution more closely resembled the expected from all possible combinations of alleles (Fig. 2C).

3.2. Aquatic mammal DQB diversity

We reviewed a total of 67 reports (Supplementary Material 1), including marine mammal MHC studies on class I and class II loci; only those with *DQB* diversity were further analyzed. Cetacea is the most studied group to date, and in this group Odontoceti species were the most studied. Fewer reports are found from Pinnipeda species, and Odobenidae only has a single report.

Overall, we were able to compile 170 and 38 unique *DQB* alleles from Cetacea and Pinnipeda, respectively. Number of alleles in Cetacea ranged from one, in *Phocoena sinus* (Munguia-Vega et al., 2007) and *Monodon monoceros* (Murray et al., 1995), to twenty two in *Balaenoptera musculus* (Moreno-Santillán et al., 2016). In Pinnipeda this number ranged from two, in *Phocarctos hookeri* (Lento et al., 2003; Osborne et al., 2013) and *Mirounga angustirostris* (Hoelzel et al., 1999; Weber et al., 2004), to eight, in *Mirounga leonina* (Hoelzel et al., 1999) and *Zalophus californianus* (Bowen et al., 2002). Sample size did not correlate to number of alleles using the whole dataset (Spearman correlation R = 0.3, P = 0.09; Fig. 3A), but this correlation was positive in Cetacea studies (R = 0.6, P = 0.007; Fig. 3B); removal of the outlier Phocidae study did not result in significant correlation for the whole dataset or Pinnipeda (data not shown). Overall, Pinnipeda studies reported lower mean number of alleles (Fig. 3C). Studied aquatic mammals were categorized across five IUCN red list categories, whereas *DQB* diversity did not seem to be greater in less threatened species (however the small number of reports in some categories precluded statistical comparisons; Fig. S1, Supplementary Material 3). Aquatic mammals that inhabit freshwater had an overall greater number of alleles when compared to exclusively marine mammals (mean allele number 9 ± 3.07 vs 5.45 ±
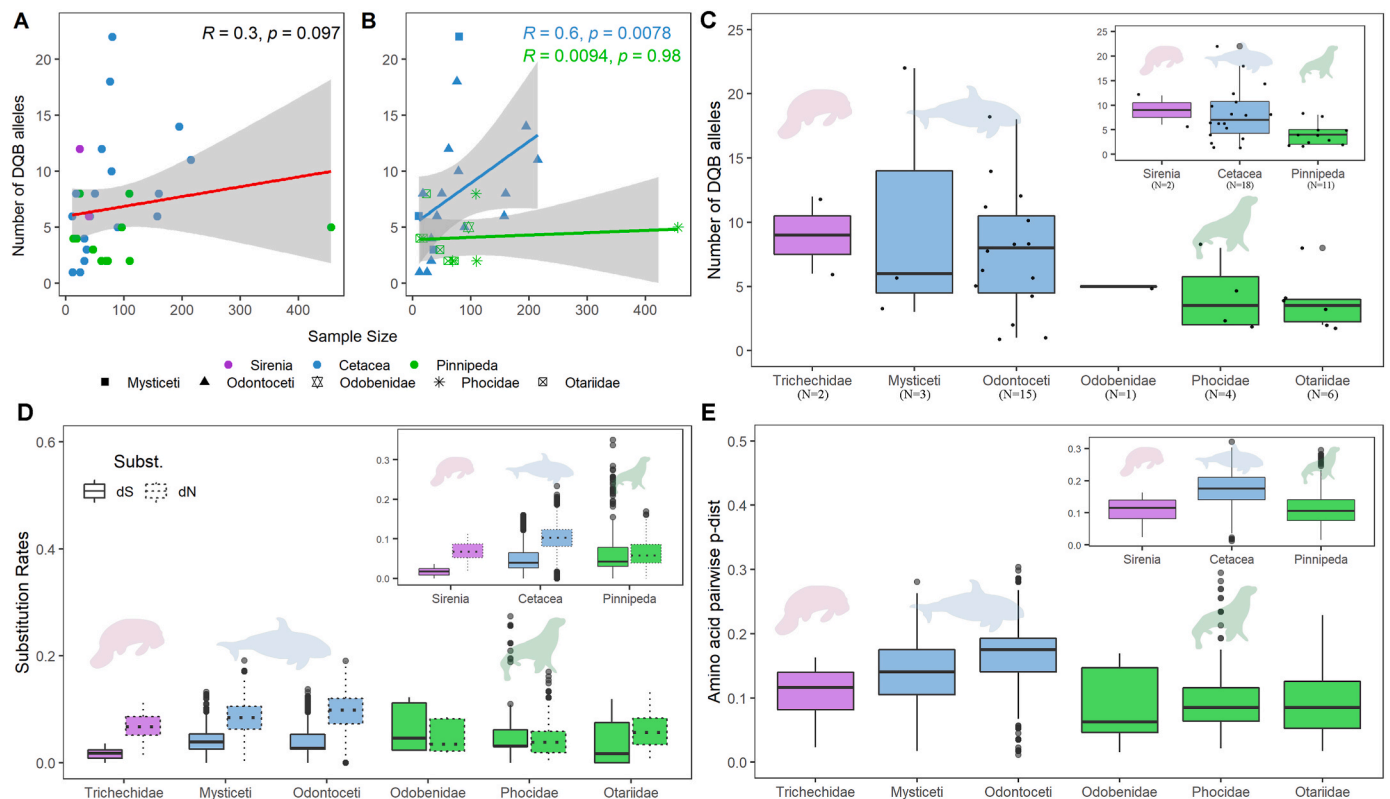

Fig. 3. *DQB* allele diversity across clades and subclades of aquatic mammals. **A)** Correlation between sample size and number of *DQB* alleles in studies on *DQB* diversity using the whole dataset and **B)** using Cetacea (blue) and Pinnipeda (green) only; shaded in gray is the 95% confidence interval. **C)** Number of *DQB* alleles. **D)** Rates of synonymous (dS) and non-synonymous (dN) substitutions. **E)** Amino acid pairwise p-distances.

5.43, Wilcoxon-Mann-Whitney p = 0.0034, Fig. S2, Supplementary Material 3).

Cetacea and Sirenia *DQB* alleles showed higher dN rates compared to dS (Fig. 3D). In contrast, Pinnipeda dS and dN were similar across all subclades, including the highest rates of synonymous substitutions (Fig. 3D). The dN/dS ratios in the Sirenia and Cetacea dataset were positive and significant for the whole exon 2 and PBS positions (Table 2), suggesting positive selection. In contrast, Pinnipeda dataset assumed ratios close to 1, including in PBS (Table 2). RELAX test showed a relaxation/intensification parameter K = 0.85, suggestive of relaxation in selection across Pinnipeda alleles, however it was not significant (p = 0.35).

We found a total of 161 unique Cetacea DQB predicted amino acid sequences, including 51/89 polymorphic positions (57.3%). Across 34 Pinnipeda unique DQB amino acid sequences, only 38/89 sites were polymorphic (42.7%). Polymorphic positions across marine mammal DQB protein sequences were similar (Fig. S3, supplementary material 3).

P-distance of predicted amino acid sequence of *DQB* alleles had similar distribution across marine mammals (Fig. 3E). Cetacea had the highest p-distance across DQB alleles compared to other marine mammal clades (P < 0.01), whereas Sirenia and Pinnipeda had similar values (P = 0.35; Table S3, Supplementary material 3). Most subclade pairwise comparisons showed significant differences in p-distance distribution, except between all Pinnipeda subclades, and between Trichechidae and Odobenidae alleles (Table S4, Supplementary material 3).

4. Discussion

4.1. MHC diversity in manatees

To our knowledge, we have showed here the first results on Sirenia MHC *DQB* diversity, including samples from two species. Despite smaller and more restricted geographic sampling, Amazonian manatees possessed greater number of alleles, haplotype and nucleotide diversity compared to West Indian manatees. Due to a dearth of sample, comparisons between manatee populations should be cautionary. However, the greater genetic diversity found in *T. inunguis DQB* agree with results from other nuclear and mitochondrial markers (Hernández Martínez et al., 2013; Hunter et al., 2012; Luna et al., 2021; Luna, 2013; Nourisson et al., 2011; Satizábal et al., 2012; Vianna et al., 2006). Despite the low genetic diversity in *T. manatus*, our results suggest the maintenance of adaptive/functional diversity.

Evidence of overall positive selection in manatee exon 2 of an MHC class II gene is highly concordant with reports from other vertebrates, including marine mammals (Cammen et al., 2015; Moreno-Santillán et al., 2016; Sonsthagen et al., 2014; Zhang et al., 2016). The prevalence of non-synonymous substitutions suggests a historical pressure for the maintenance of divergent *DQB* proteins in the population by means of balancing selection. Interestingly, *T. inunguis* individuals seem to retain more divergent alleles (Fig. 2C), which might corroborate with the divergent allele hypothesis (Wakeland et al., 1990). This hypothesis postulates that populations and individuals with divergent alleles will have increased fitness because they can present a broader arrange of antigens. Further investigations are needed to directly test this hypothesis in manatee populations.

The lack of stop codons and maintenance of conserved domains on all alleles of both species suggests they are likely expressed *DQB* genes. The Florida manatee (*T. manatus latirostris*) genome possesses four *DQB* loci, located in two scaffolds, three of which are pseudogenes (Sá et al., 2019). Among pseudogenes, only one had stop codons in the exon 2. Other pseudogenes, despite not having stop codons in the exon 2, have several mutations in this exon (including in non-PBS residues), resulting in long branches in phylogenetic analysis when compared to functional gene sequences (data not shown), different from the alleles presented

here. The primers designed in this study have only two Primer-BLAST hits in the Florida manatee genome (the functional gene and one pseudogene). The primer pair had three to four mismatches to the pseudogene and the expected PCR product size is 8-bp smaller than the sequences amplified in this study. In addition, the presence of four alleles in a single individual suggests that at least some haplotypes have duplicated loci. Therefore, we believe alleles are from functional *DQB* loci and not from pseudogenes, and copy number variationfundin may increase the number of expressed genes in manatees.

The occurrence of four identical alleles in *T. manatus* and *T. inunguis* samples suggests trans-species polymorphism. This phenomenon is believed to be a result of old allelic lineages maintained by balancing selection in ancestral species prior and throughout the speciation divergence (Klein et al., 2007; Těsický and Vinkler, 2015). Among the four alleles shared by both manatee species, all occur in Brazilian West Indian manatees, while Florida manatees lack two of them. Since the species may hybridize in northern Brazil (Garcia-Rodriguez et al., 1998; Vianna et al., 2006), it is difficult to differentiate between trans-species polymorphism and genetic introgression.

Despite the identification of later generation hybrids between both manatee species in French Guiana (Vilaça et al., 2019), Luna et al. (2021) did not find evidence of a hybrid swarm in this region using a robust dataset and well-suited microsatellite markers (Lima et al., 2019; Vilaça et al., 2019). Nonetheless, irrespective of the degree that manatees undergo hybridization in the sympatric region, historical (and even rare) events of past hybridizations may generate islands of introgressed genetic diversity in species' genomes (Gokcumen, 2020; Martin and Jiggins, 2017), and the MHC loci, evolving under balancing selection, may be particularly prone to such phenomenon (Dudek et al., 2019; Hedrick, 2013) and has been reported in other vertebrates (Angelone et al., 2018; Dudek et al., 2019; Grossen et al., 2014; Sagonas et al., 2019; Wegner and Eizaguirre, 2012).

Possible explanations for increased *DQB* diversity in Brazil compared to Florida *T. manatus* may be due to: differences in sampling effort; historical population sizes and demography (correlating to the overall reduced genetic diversity found in Florida possibly due to founding effect (Garcia-Rodriguez et al., 1998; Tucker et al., 2012; Vianna et al., 2006)); and historical introgression contributing to diversity (supported by the fact that two out of three unique alleles to Brazilian *T. manatus* are identical to *T. inunguis* alleles, Table S2, Supplementary Material 3). Current understanding of manatee evolution places the ancestor of *Trichechus* on the Amazon basin (de Souza et al., 2021). Therefore, increased diversity found in *T. inunguis* may be due to larger long-term effective population size of this species, as speciation and migration of *T. manatus* may have imposed bottlenecks that resulted in loss of genetic diversity from ancestral populations.

Future studies are needed to understand the evolutionary history of MHC genes from manatees, including greater sampling from all three manatee species, including other *T. manatus* populations (e.g. from other Central and South American countries, the Caribbean islands, Florida), and from the sympatric region. Noteworthy, sequencing of multiple sites (i.e. other exons, introns and/or linked loci) of shared alleles may be needed to differentiate trans-species polymorphism from potential introgression.

4.2. Aquatic mammal DQB evolution

Manatee number of alleles lie within the distribution of reported marine mammals. The number of alleles found in *T. inunguis* is similar to those reported by Cammen et al. (2015) and Pagán et al. (2018) for *Tursiops truncatus*, despite the smaller sample size in the present study. West Indian manatees have a greater *DQB* diversity than *Balaenoptera physalus* and *Sousa chinensis*, two marine mammal species with similar sampling size to our data (Nigenda-Morales et al., 2008; Zhang et al., 2016). As a matter of fact, only three Cetacea species had more than eight *DQB* alleles (Arbanasić et al., 2014; Cammen et al., 2015; Du et al.,

2010; Moreno-Santillán et al., 2016; Pagán et al., 2018; Xu et al., 2007), and in some cases despite great sampling efforts (Hayashi et al., 2006; Heimeier et al., 2018; Murray et al., 1999); eight alleles was the upper limit for Pinnipeda diversity (Bowen et al., 2002; Hoelzel et al., 1999). Population size and historical demographic fluctuations are likely to impact the number of alleles found, however aquatic mammals currently under lower levels of threat does not seem to possess more alleles than more threatened species (Fig. S1, Supplementary Material 3).

Comparisons between ours and others' studies should take into consideration the amplification methods used. Most studies report only 172bp of Cetacea *DQB* alleles or even smaller fragments from most Pinnipeda, potentially leading to the *DQB* diversity being under-estimated. In fact, several amplification assays used for marine mammals have one or both primers annealing in predicted PBS sites (Fig. S4, Supplementary Material 3) which are the most polymorphic positions of MHC genes and may have key differences at the 3′of the primer and could result in failure of some allele amplification. Regarding our characterization of manatee *DQB* diversity, the lack of other primer sets precludes a definite conclusion regarding *DQB* allele number or copy number variation; however, we believe this has little effect on the main conclusions drawn from the comparisons to other marine mammals, as our main analysis is based on molecular evolution statistics (such as dN/dS and p-distance), less sensible to incomplete polymorphism characterization. Despite these shortcomings, our results point to a more complex scenario of marine mammal MHC evolution than suggested previously.

Slade's (1992) hypothesis – that marine mammals experience reduced pathogen pressure on aquatic environments which results in reduced MHC diversity – was initially supported by several reports on low genetic diversity in Cetacea and Pinnipeda (e.g. (Murray and White, 1998; Slade, 1992; Trowsdale et al., 1989; Weber et al., 2004)). Additionally (Villanueva-Noriega et al., 2013), provided the first direct test of Slade's (1992) first prediction by comparing *DQB* diversity of terrestrial mammals and cetaceans, suggesting weaker balancing selection on the later. As suggested by Slade (1992), it would be expected that marine mammals that are not fully aquatic would be subject to greater pressure from pathogens from terrestrial environments and other terrestrial mammals. Also, Pinnipeda species with high density colonies would also provide a well-suited condition for infectious diseases to spread.

Contrary to expectations, substitution rates point to a distinct evolutionary history in Pinnipeda *DQB*, with alleles showing greater synonymous substitutions than expected. The accumulation of synonymous substitutions may reflect a relaxed selective pressure in the locus, as suggested by the close to unity values found in the exon 2 dN/dS ratios. Relaxation/intensification index K showed a smaller then 1 value for Pinnipeda *DQB*, compatible with relaxation in the selection in this branch, although results were not significant, possibly due to the small number of codons tested, especially in the Pinnipeda dataset. This contradicts Slade's (1992) prediction that Pinnipeda would be subject to greater selective pressure than other marine mammals due to greater exposure to terrestrial mammals' pathogens.

Few studies reported substitution rates in Pinnipeda *DQB*, including small rates of non-synonymous substitutions in *Odobenus rosmarus* (Sonsthagen et al., 2014), but not in *Neophoca cinerea* (Lau et al., 2015). Sirenia and Cetacea *DQB* have overall greater diversity, and substitutions rates that better resemble those found in classic MHC genes from terrestrial mammals. Sirenia *DQB* had the highest dN/dS ratio, but this may be a consequence of the reduced number of sequences and taxa included in this group. Interestingly, the accumulation of synonymous substitution in Pinnipeda does not have a pronounced effect on amino acid diversity, since we found similar distribution of amino acid p-distance comparing the three clades. The greater values observed in Cetacea p-distances may stem from the greater number of taxa and sequences included in the analysis.

The characteristics found here for Pinnipeda *DQB* do not seem to reflect the overall evolution of Carnivora *DQB*, as high number of alleles and/or high ratio of dN/dS has been found in terrestrial carnivores, such as dogs (Wagner et al., 1998), wolves (Seddon and Ellegren, 2002), Japanese black bears (Yasukochi et al., 2012), brown bears (Kuduk et al., 2012), and giant panda (Chen et al., 2009). *DQB* from carnivore lineages closer to Pinnipeda are less studied. To our knowledge, only two studies described (low) *DQB* diversity in the sea otter and European badgers (Bowen et al., 2006; Sin et al., 2012), but small sample size (≤7 individuals) prevents to draw meaningful conclusions based on their *DQB* polymorphism. Therefore, Pinnipeda *DQB* may be evolving under relaxed balancing selection since its split from other carnivore lineages. Interestingly, De Assunção-Franco et al. (2012) reported association of *DQB* genotypes to mortality in grey seals, despite low allele number in the species. Therefore, DQB may still impact fitness, despite evolving under weaker selective pressure, which helps in explaining the maintenance of diverse amino acid composition found in our results. This association may also be due to linked loci in the MHC region, as *DRB* has also been associated with fitness and disease in Pinnipeda (Aceve-do-Whitehouse et al., 2018; Bowen et al., 2005; Lenz et al., 2013), and shows overall "more classical nature" compared to DQB due to greater polymorphism (Bowen et al., 2004; Lenz et al., 2013).

These results may reflect a distinct evolution of Pinnipeda *DQB*, despite being considered a class II MHC classical gene. Several vertebrate lineages have undergone distinct evolutionary paths, including loss of function of DQ and DP genes and expansion of DR in felines (Yuhki et al., 2003), loss of DR and expansion of DP in mole rats (Nizetic et al., 1987), loss of class II in Gadiformes (Malmstrøm et al., 2016; Star et al., 2011), pipefishes (Haase et al., 2013; Small et al., 2016) and Lophioidei (Dubin et al., 2019), probable loss of functional DP in Cetacea, Pinnipeda and in the tenrec, and loss of functional DQ in Afrosoricida (Sá et al., 2019). Differences in evolutionary trajectories across clades makes it difficult to address ecoevolutionary questions such as the evolution of MHC in aquatic mammals.

The MHC evolution shows great plasticity in accommodating distinct genomic background related to a species ecoevolutionary trajectory, probably because there is some degree of redundancy in the function of classical genes. Despite this, aquatic mammals immunogenetic studies have largely focused on characterizing *DQB* diversity, and therefore caution is needed when interpreting previous reports and our results as representative of the evolution of the MHC as a whole. Not only *DQB* itself does not represent the whole DQ molecule diversity (of which binding groove includes polymorphic residues from both DQB and DQA molecules), but also other closely linked polymorphic MHC loci may play a role in disease resistance, such as DR, DP, in addition to classical class I molecules. Therefore, a deeper understanding of aquatic mammal MHC evolution will come with greater sampling efforts and population level characterization of diversity of additional loci, such as other classical class II and class I genes. As for now, the reduced number of studies using other loci in all three lineages makes it difficult to perform similar analysis as the one presented here for *DQB*. Sirenia would benefit from greater efforts in characterizing its MHC diversity and function, not only in manatees but also in dugongs. As for today, little is known regarding MHC diversity and function in general in Afrotherians, with only a few reports on elephants and mammoth *DQA* polymorphism (Archie et al., 2010; Pečnerová et al., 2016). Therefore, any immunogenetic comparison among aquatic mammals will bias analysis if based on the assumption that the immune response in these three clades follow the same rules.

Noteworthy, Slade's (1992) hypothesis did not address any possible differences between freshwater and marine pathogen environmental pressures. Animals inhabiting freshwater bodies may be subject to distinct evolutionary pressures, when compared to marine environments, due to distinct microbial diversity, diet, and seasonality (Tee et al., 2021; Wilhelm and Matteson, 2008). Our results points to a greater number of *DQB* alleles in aquatic mammals inhabiting

freshwater compared to exclusively marine mammals. However, two species (*Neophocaena phocaenoides* and *T. truncatus*) are overrepresented in the freshwater inhabiting group in our analysis. Therefore, this result is preliminary and should be interpreted with caution. Antillean manatees may provide a suitable model for understanding these differences, as recent evidence suggests distinct ecotypes of Antillean manatees comparing riverine and coastal marine populations (Castelblanco-Martínez et al., 2021). In fact, Slade's hypothesis also did not address any possible differences between coastal and marine habitats, as coastal environments may be more subject to input from terrestrial habitats due to run offs. Future research is needed to elucidate how and whether possible differences between the diverse environments aquatic mammals live in influences on their MHC evolution.

Taken together, our results point to the maintenance of functional/adaptive genetic diversity in both species of manatees in the Americas, however reduced genetic diversity in *T. manatus* is still of concern. Balancing selection likely shaped the diversity of manatees, with several positively selected codons. Allele composition suggests trans-species polymorphism between both species but cannot rule out sharing of alleles through introgression. Fully aquatic mammals do not have lower *DQB* diversity compared to Pinnipeda, which contradicts Slade's (1992) second prediction. However, more studies from Sirenia and Pinnipeda DQB, and other classical class I and II loci from all aquatic mammals are needed to further elucidate this prediction. In addition, the scarcity of Pinnipeda reports hinders testing Slade's (1992) third prediction, regarding distinct pressure on Pinnipeda species that spend more time ashore and in colonies closer to terrestrial mammal populations. Based on the data presented here, the hypothesis that aquatic mammals MHC are under weaker selective pressure is not supported and point to the need to refine other possible ecoevolutionary processes responsible for the trends observed in Cetacea and Pinnipeda *DQB* repertoire.

Ethics statement

All procedures were approved by the Ethics Committee of Scientific Research of Universidade Federal do Pará (Permit CEPAE 68–2015), sample collection in Brazil was performed by trained personnel under the SISBIO 50641-2 license, and sample exportation to United States was approved under CITES Export Permit 17BR023548/DF and CITES Import Permit 16US808447/9. Live animals were handled by authorized and trained personnel. West Indian manatees from Florida, Belize and three from Brazil were from USGS sample bank are under USGS Sirenia Project's Federal Wildlife Research Permit MA791721-5, Brazilian are samples under CITES Export Permit 09BR003661/DF and Import Permit 08US808447/9.

Data accessibility

Manatee *DQB* allele sequences have been uploaded to NCBI under Genbank accessions MZ342788-MZ342805. Datasets and R scripts used for this article are available at Figshare (Sá et al., 2021). The references, methods and results supporting this article have been uploaded as part of the supplementary material.

Declaration of competing interest

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2022.104398.

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