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Whole genomes reveal multiple candidate genes and pathways involved in the immune response of dolphins to a highly infectious virus

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Abstract

Wildlife species are challenged by various infectious diseases that act as important demographic drivers of populations and have become a great conservation concern particularly under growing environmental changes. The new era of whole genome sequencing provides new opportunities and avenues to explore the role of genetic variants in the plasticity of immune responses, particularly in non-model systems. Cetacean morbillivirus (CeMV) has emerged as a major viral threat to cetacean populations worldwide, contributing to the death of thousands of individuals of multiple dolphin and whale species. To understand the genomic basis of immune responses to CeMV, we generated and analysed whole genomes of 53 Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) exposed to Australia's largest known CeMV-related mortality event that killed at least 50 dolphins from three different species. The genomic data set consisted of 10,168,981 SNPs anchored onto 23 chromosome-length scaffolds and 77 short scaffolds. Whole genome analysis indicated that levels of inbreeding in the dolphin population did not influence the outcome of an individual. Allele frequency estimates between survivors and nonsurvivors of the outbreak revealed 15,769 candidate SNPs, of which 689 were annotated to 295 protein coding genes. These included 50 genes with functions related to innate and adaptive immune responses, and cytokine signalling pathways and genes thought to be involved in immune responses to other morbilliviruses. Our study characterised genomic regions and pathways that may contribute to CeMV immune responses in dolphins. This represents a stride towards clarifying the complex interactions of the cetacean immune system and emphasises the value of whole genome data sets in understanding genetic elements that are essential for species conservation, including disease susceptibility and adaptation.

KEYWORDS
cetacean morbillivirus, ecological genomics, immune genes, inshore dolphin, whole genome sequencing, wildlife disease

1 | INTRODUCTION

Climatic variations, natural and anthropogenic alterations to ecosystems, changes in host behaviour, and the movement of pathogens and vectors have all contributed to the emergence of infectious diseases in wildlife populations (Cunningham et al., 2017; Morens et al., 2004; Titcomb et al., 2019; Williams et al., 2002). Infectious diseases have become a major conservation concern due to the ability of pathogens to rapidly evolve, their short generation times and often complex transmission dynamics, as well as being able to cause swift and widespread mortality, diminish genetic diversity and contribute to population declines and extinctions (Altizer et al., 2003; Blanchong et al., 2016; Stejskalova et al., 2017). Disease outbreaks are beginning to become a cause for concern in cetacean populations worldwide, especially for species that exhibit high social connectivity and gregarious behaviour, and for populations that are immunologically naïve, small, threatened, or immune-suppressed (Gulland & Hall, 2007; Van Bressem et al., 2009; Weiss et al., 2020). In recent years, the reporting of infectious disease and strandings in cetaceans has increased, with one highly contagious and virulent pathogen emerging as a major threat to their populations; cetacean morbillivirus (CeMV) (Sacristán et al., 2015). CeMV belongs to the genus *Morbillivirus*, which affects both terrestrial mammals (humans [measles virus], canines [canine distemper virus], cattle [rinderpest virus], goats and sheep [peste des petits ruminants virus], and two novel morbilliviruses in cats and bats) and marine mammals (true seals [phocine distemper virus] and cetaceans) (Alfonso et al., 2016; Ohishi et al., 2019). These viral species are distinct, but share a common phylogenetic origin, similar genome structure, symptoms of infection and pathomorphology (da Fontoura Budaszewski & von Messling, 2016; Diaz-Delgado et al., 2019). This, along with observed cross-species transmissions (Jo et al., 2018; Padalino et al., 2019; Stejskalova et al., 2017), suggests that knowledge gained on immune responses for one viral species may be applicable more generally to morbilliviruses.

Since its discovery in the late 1980 s, CeMV has become a great conservation concern given the increased reporting of unusual mortality events (i.e., unexpected and significant die-offs of a marine mammal population that warrants a rapid response by managers (Kemper et al., 2016; MMPA, 1972)) in a larger number of known host species and populations (Di Guardo et al., 2005; Van Bressem et al., 2009). The virus has been implicated in the death of tens of thousands of cetaceans worldwide (Van Bressem et al., 2014), but until recently had only been recognised as a contributing factor in the death of a small number of bottlenose dolphins (*Tursiops* spp.) across Australia (Stephens et al., 2014; Stone et al., 2011, 2012). However, in 2013 CeMV was identified in dolphins that died during an unusual mortality event involving at least 50 individuals of three species (Indo-Pacific bottlenose dolphin, *Tursiops aduncus*; common bottlenose dolphin, *T. truncatus*; and common dolphin, *Delphinus delphis*) in South Australia, becoming the largest confirmed CeMV outbreak in Australia and the first recorded deaths from CeMV in this state (Kemper et al., 2016). This unusual mortality event lasted approximately seven months (March to September), and the initial months of the outbreak coincided with climatic anomalies that resulted in abnormally high sea surface temperatures (Kemper et al., 2016). Indo-Pacific bottlenose dolphins were the most affected, with 31 testing genetically positive for the virus, and majority of these were neonates, calves, and juveniles. A total of 29 were from Gulf St Vincent (GSV), a population that is relatively small (700–1,200 dolphins, Bilgmann et al., 2019), exhibits high social connectivity (Zanardo et al., 2016), shows relatively low genetic diversity (Pratt et al., 2018), and may be considerably vulnerable to epizootic events (Reed et al., 2020). These characteristics, coupled with the CeMV-related mortality event, provides a unique opportunity to understand the importance of host genetic factors affecting disease susceptibility in cetacean species.

Host genetic factors are known to be key drivers in the plasticity of immune responses in natural populations, being major determinants of an individual's susceptibility ("the state of being very likely to be influenced, harmed or affected by something" [Susceptibility, 2021]) and resistance ("the ability not to be affected by something, especially adversely" [Resistance, 2021]) to infection (Karlsson et al., 2014; Stejskalova et al., 2017). For example, inbreeding can reduce fitness through homozygosity in deleterious recessive alleles, a lack of genetic diversity can reduce adaptive potential, and homozygosity in immune-related genes may hinder pathogen recognition (Blanchong et al., 2016; Smith et al., 2009). Yet studies investigating the role of host genetic factors in disease susceptibility and resistance in wildlife populations are relatively limited. Association-based studies provide a favourable framework for identifying associations between genomic locations, regions or genes, and complex traits in natural populations. Studies addressing the role of host genetics in combating infection have generally targeted a small number of genomic regions of known functional importance, and genes with strong effect. For example, the major histocompatibility complex (MHC) are among some of the most targeted and well-studied immune associated genes in model and non-model species, including cetaceans (Acevedo-Whitehouse & Cunningham, 2006; Cammen et al., 2015; Elbers et al., 2018; Manlik et al.,. 2019; Martin & Carrington, 2005; Pagan et al.,. 2018). For example, by comparing two populations of Indo-Pacific bottlenose dolphins with varying levels of reproductive output and population viability, Manlik et al. (2019) found that the population with low reproductive output had lower levels of MHC diversity and therefore was possibly at greater risk of succumbing to human induced pressures. In the case of immune responses to the measles virus, specific alleles within the human leukocyte antigen (HLA) genes class I and II (*B*; *DQA*, *DQB*, *DRB*) have been associated with varying antibody titers following vaccination against the virus (Haralambieva et al., 2015). However, many other non-MHC genes have been proposed to be involved in host immune responses to morbilliviruses. For example, the signalling lymphocyte activation molecule (SLAM) has been identified as an immune cell receptor for measles, canine distemper, rinderpest and peste des petitis ruminants viruses, and is suggested to be a universal receptor for entry and propagation of morbillivirus in all mammals, including cetaceans

(Melia et al., 2014; Ohishi et al., 2019; Sato et al., 2012; Shimizu et al., 2013). Other genes, including viral binding genes, cytokine receptor genes, pathogen-associated sensing genes and antiviral genes were also suggested to be involved in immune responses to morbilliviruses (Hashiguchi et al., 2011; McCarthy et al., 2011; Haralambieva et al., 2015; Stejskalova et al., 2017).

Advancements in next generation sequencing, computational power and improved availability of genomic data has enabled the move from a targeted to a nontargeted approach of association-based studies. This approach enables the search for multiple genetic variants across the genome under selection and associated with a trait, without the need of prior knowledge. This framework is frequently utilised in humans, model organisms and agricultural systems (Elbers et al., 2018), and while still limited, advancing technologies have now enabled association studies in wildlife populations. In particular, whole genome data has been utilised to investigate immune responses of endangered and vulnerable Australian marsupials (Tasmanian devil, *Sarcophilus harrisii*; and the koala, *Phascolarctos cinereus)* to two highly damaging diseases that continue to threaten populations across their distribution (Johnson et al., 2018; Wright et al., 2017). The move to nontargeted approaches and large genomic data sets improves our ability to address the genetic basis of adaptation in wildlife populations and allows us to understand the role that genetic variants play in the plasticity of immune responses, and in the susceptibility of individuals and populations to infectious diseases.

In this study, we expand substantially on previous work based on reduced representation sequencing (RRS) (Batley et al., 2019) to investigate the genomic basis of resistance and susceptibility of Indo-Pacific bottlenose dolphins to CeMV using whole genomes. Using a much larger data set, we searched for regions of the genome under selection between case (nonsurvivors) and control (survivors) from the viral outbreak to identify genetic variants, genes and pathways associated with resistance and susceptibility to CeMV. Our study provides the first whole genome-based information to enable the screening of other cetaceans for potential genetic risk factors, ultimately enabling the identification of populations and species particularly vulnerable to large-scale CeMV outbreaks.

2 | MATERIALS AND METHODS

2.1 | Study species, sites and sample collection

Indo-Pacific bottlenose dolphins that died and stranded during an unusual mortality event throughout South Australia between March and September 2013, were collected by the South Australian Museum for post-mortem examinations. Histopathological examinations, reverse-transcription polymerase chain reaction (RT-PCR) and/or immunohistochemical assays confirmed that CeMV infection and related pathologies were the main contributing factor in the dolphin deaths (Table S1 and see Kemper et al., 2016). Muscle tissue from 29 Indo-Pacific bottlenose dolphins from GSV and adjacent waters, and one from Spencer Gulf that died during the unusual mortality event, tested positive for CeMV and generally exhibited CeMV related pathologies (e.g., pneumonia with syncytial cells, lymphoid depletion with systemic secondary infection by bacteria, fungi, or parasites) were provided by the South Australian Museum and formed the case group. Case samples were classified into age classes, with the majority of the strandings being young dolphins (neonates, calves, and juveniles: <1.6 m in length) (young n = 28, adults n = 2). These samples were frozen at −80˚C and kept at the South Australian Museum before being transferred to 90% ethanol and to the Molecular Ecology Laboratory at Flinders University (MELFU).

Samples were complemented with biopsy samples from free-ranging Indo-Pacific bottlenose dolphins from GSV and adjacent waters collected between 2014 and 2015 (Bilgmann et al., 2007; Pratt et al., 2018; Zanardo, Parra, et al., 2016), which putatively survived the outbreak (i.e., control samples). These skin and blubber samples were collected using either the PAXARMS biopsy system (Krützen et al., 2002) or a hand-held biopsy pole (Bilgmann et al., 2007). Age classes (calves, juveniles, and adults) of sampled individuals were estimated in situ based on body size and association with an adult dolphin (see Zanardo, Parra, et al., 2016 for details). This resulted in a total of 34 control samples, with samples from young (n = 11) complemented with random adult samples from GSV and close adjacency (n = 23). These control samples are considered putative survivors since they belong to the same genetic and socially cohesive population (Pratt et al., 2018; Zanardo, Parra, et al., 2016) as the one most impacted during the unusual mortality event and were collected within 18 months of the outbreak. Biopsy samples were preserved in a salt-saturated solution of 20% dimethyl sulphoxide (DMSO) and stored at −80°C at the MELFU. Dolphins were genetically sexed using a polymerase chain reaction (PCR) (Banks et al., 1995). The phenotypic data for samples that passed DNA quality controls and were subsequently selected for whole genome sequencing is available in Table S1.

2.2 | DNA extractions and whole genome sequencing

Genomic DNA was isolated from control samples following the salting out method (Sunnucks & Hales, 1996), while genomic DNA was extracted from case samples using the Qiagen DNeasy blood and tissue kit following the manufacturer's protocol. The purity of extractions was verified using a ND-1000 spectrophotometer (Nanodrop; Thermo Scientific), and quantity assessed using a fluorometer (Qubit; Life Technologies). The DNA integrity was further assessed by gel electrophoresis (2% agarose gels, produced in-house). All extractions were expected to pass quality controls based on standards set by the Australian Genome Research Facility (AGRF), where libraries were prepared and sequenced. Specifically, samples were required to have a quantity ≥20 ng/µl, a high molecular weight (≥20 kb), free of RNA (assessed on agarose gels) and an A260/280 (protein contamination) ratio

between 1.8 and 2.0. Extractions that did not pass quality controls were re-extracted a maximum of three times, using the same method but with altering amounts of tissue to potentially increase the concentration and improve the quality of DNA. As expected, extractions that failed the quality controls were typically of case samples, since these were obtained from carcasses rather than free-ranging dolphins. Case samples with a low concentration for all extractions were then combined, and concentrated using a centrifuge vacuum concentrator (Hetovac; Heto Laboratory). Extractions from 53 samples that passed all quality controls (case, n = 19 and control, n = 34) were subsequently selected for library preparation and whole genome sequencing at AGRF (Table S1). Libraries were prepared using the NEBNext Ultra II DNA library prep kit and sequenced on two lanes of the Illumina NovaSeq 6000 S2 platform (150 bp PE). Samples were sequenced at ~7x coverage, excluding one sample from GSV that was sequenced at a higher depth of coverage (~28×) to form a reference genome (Batley et al. in preparation). While throughout this study we refer to the species as the Indo-Pacific bottlenose dolphin (*T. aduncus*), it has been previously suggested to represent a separate species, endemic to southern Australian waters, *T. australis* (Charlton-Robb et al., 2011). However, recent studies suggest this is more likely to be a subspecies of *T. aduncus* (Moura et al., 2020), and therefore we refer to the reference genome here as the southern Australian bottlenose dolphin (SABD). Details regarding the construction, quality and statistics of this reference genome will be available in Batley et al. (in preparation).

2.3 | Read processing, SNP calling and filtering

Raw sequencing data was preprocessed following the pipeline adapted from GATK best practices (Van der Auwera et al., 2013), with modifications. Firstly, reads were trimmed if read quality was below 23 in a sliding window of five nucleotides, while adapters were removed using Trimmomatic v0.38 (Bolger et al., 2014). The remaining reads were mapped to the chromosome-length scaffolded *S*ABD reference genome using Bowtie2 v2.2.7 (Langmead & Salzberg, 2012). The resulting SAM files were then converted to BAM files, duplicate marked and sorted using Picard (Picard Toolkit, 2019). Indels were then locally realigned to correct mapping errors using GATK before merging the replicate reads from different libraries with samtools (Li et al., 2009).

SNPs were called from the mapped reads of all individuals using the SABD reference genome in a two-part process using bcftools (Li, 2011). This involved generating genotype probabilities at each genomic position before calling the SNPs. SNPs were then filtered with vcftools (Danecek et al., 2011) and using parameters described in Brauer et al. (2016). In short, reads with a minor allele frequency <3% and genotyped in <80% of the samples were excluded. Indels were removed and only SNPs with a quality and depth ratio of 2%, mapping quality >30 and mean depth <12 were retained. Finally, Hardy-Weinberg equilibrium was calculated within the two groups (cases and controls), and SNPs that were out of HWE in each group were excluded. SNPs were called altogether, including those from available whole genomes of common dolphins and common bottlenose dolphins (data not presented here), but as this study focused on Indo-Pacific bottlenose dolphins from southern Australia, only SNPs that are unique to this lineage were retained (see Table S2 for SNPs retained at each step).

2.4 | Whole genome association study

2.4.1 | Potential effects of inbreeding, relatedness, sex, and age-classes

As inbreeding can reduce disease resistance due to the loss of genetic diversity (Acevedo-Whitehouse et al., 2003), levels of inbreeding were calculated to test for potential effects of inbreeding. The inbreeding coefficient, F, was calculated within and between cases and controls using the het command in Plink v1.9, based on an unlinked SNP data set (189,178 SNPs). The mean F of each group was compared using an independent samples t test.

Levels of relatedness within and between cases and controls as well as differences in the representation of sexes and age classes between groups were also calculated to assess the potential influence of these factors on the outcome of an individual. Pairwise relatedness between individuals based on the unadjusted Ajk statistic method of Yang et al. (2010) was estimated using vcftools. Pairwise relatedness within and between groups, as well as the mean number of individuals of each sex and age class between groups were then compared using an independent sample t test.

2.4.2 | Identifying SNPs under selection

Allele frequency differences between the two groups were calculated to identify SNPs potentially involved in resistance or susceptibility. This analysis used 7,720,686 SNPs and was based on two association tests implemented in Plink v1.9; the chi-square test and Fisher's exact test. SNPs with a highly significant p-value ($p \leq .001$) were selected as outlier SNPs, as per Batley et al. (2019). These two tests were complemented by the Weir and Cockerham's F_{ST} (Weir & Cockerham, 1984), which estimates differentiation between groups based on allele frequency shifts. F_{ST} was calculated between cases and controls using the –weir-fst-pop command in vcftools. SNPs with an F_{ST} value greater than five standard deviations from the mean (0.0024 ± 5SD) were selected as outlier SNPs (Axelsson et al., 2013; Kardos et al., 2015). Outlier SNPs from each of the three tests were compared, and those identified as outliers in at least two tests were selected as candidate SNPs to reduce false positives. The two tests implemented in Plink also output odds ratios (OR), which were used to test the odds of the minor allele being in association with an outcome (i.e., nonsurvival).

2.4.3 | Annotation of candidate SNPs

To annotate and explore the function of candidate SNPs, 600 bp flanking regions of the candidate SNPs were aligned to *T. truncatus* proteins (GCF_001922835.1) using BLASTX v2.2.28. This used an alignment length of above 30 amino acids, similarity above 50%, and an e-value threshold of 8e-07. For all alignments to the proteins, the genomic region of the SNP (intronic or exonic) and their predicted functional effect (missense vs. synonymous changes) were investigated using SnpEff (Cingolani et al., 2012). Specifically, a VCF file of all candidate SNPs with flanking regions that aligned to the protein database was generated and the SNPs were annotated against the SABD reference genome. The SABD reference annotation was used here as SNPs were initially mapped to the SABD reference genome and SnpEff required knowledge on SNP location; however, the annotation was not available for the initial protein annotation. Functions of the putative candidate genes were explored using gene ontology (GO) terms provided by UNIPROTKB (UniProt, 2019), and their involvement in immune pathways and gene interactions were explored with human ENSEMBL identifiers and REACTOME (Fabregat et al., 2018).

2.4.4 | Candidate immune gene approach

Several genes potentially involved in immune responses to morbilliviruses have been proposed (Hashiguchi et al., 2011; Haralambieva et al., 2015; McCarthy et al., 2011; Stejskalova et al., 2017), but some of these genes were not identified as candidate genes in our data set. To investigate whether SNPs within these genes are in fact neutral between cases and controls, or alternatively, under selection but did not align to the protein coding regions, the allele frequency and genotype counts for each SNP within each gene were compared between cases and controls. To achieve this, each gene location was extracted from the SABD reference genome and SNPs within the specified regions were extracted using VCFTOOLS. As the SABD annotation is in a draft format, genes that were not found in the SABD annotation were downloaded from NCBI (*T. truncatus*; GCF_001922835.1) and mapped to the SABD reference genome using BLAST v2.2.28. Allele frequency differences were calculated using a chi-square test, and genotypes of the top performing SNP within each gene (i.e., SNP with the greatest differentiation between cases and controls) were counted using Plink.

3 | RESULTS

Whole genome sequencing produced a total of 4,274,472,237 reads for 53 Indo-Pacific bottlenose dolphins from South Australia (Figure 1). After quality filtering, 3,310,493,013 reads (mean = 31,231,066 ± 13,586,174) remained, of which an average of 96.81% of reads mapped to the SABD reference genome. Calling SNPs from the genome resulted in a total of 33,386,256 SNPs, of which 17,226,558 remained after quality filtering (Table S2). Of these SNPs, 10,168,981 (on 23 chromosome-length scaffolds and 77 smaller scaffolds, Figure S1) were unique to *T. aduncus*. The final data set available for analysis therefore, consisted of 10,168,981 SNPs for 53 individuals with an average of 1.02% missing data (SD ± 1.32%). Missing data did not differ significantly between cases and controls (cases = 0.95% ± 0.61%; controls = 1.06% ± 1.6%, p = .389).

3.1 | Potential effects of inbreeding, relatedness, sex, and age-classes

There was no significant difference in the mean inbreeding coefficient between the two groups (cases = 0.0574 ± 0.049; controls = 0.0289 ± 0.061, p = .087), suggesting that genome-wide levels of inbreeding did not influence susceptibility of case dolphins to CeMV during the outbreak.

The mean relatedness of pairs of individuals within and between groups was not significantly different (cases = −0.0185 ± 0.056; controls = −0.0184 ± 0.051; case–control = −0.0226 0.036; all p > .05). Likewise, there was no significant difference between the sex composition between groups (cases: M = 10, F = 9; controls: M = 21, F = 9; p = .527). There was, however, a significant difference between the representation of different age classes in the two groups, but due to the limited number of adult case samples (n = 2), the influence of age could not be accounted for in the analysis.

3.2 | Identifying SNPs under selection and annotation of candidate SNPs

Methods to detect SNPs under selection between case and control individuals identified outlier SNPs in all three tests, with 13,000 outlier SNPs detected using the Fisher's exact test, 17,398 SNPs for the chi-square test and 36,726 SNPs for the F_{ST} test of differentiation. Of these outliers, 5,105 SNPs were present in two tests, and a further 10,664 SNPs were present in all three tests. A total of 15,769 SNPs (<0.16% of all SNPs) found on 22 chromosome-length scaffolds showed putative signatures of selection between case and control individuals and were considered candidate SNPs.

Of the 15,769 candidate SNPs and associated flanking regions, 689 aligned and annotated to the common bottlenose dolphin (*T. truncatus*) proteins. These SNPs annotated to 295 protein coding genes and six uncharacterised proteins (Table S3). Investigation of all candidate genes and their involvement in different pathways found that 131 candidate genes were related to 856 different biological subpathways that can be grouped into 25 pathways (Table S4). The key pathway of interest is the immune system (37 genes) (Figure 2, Table S4), however other pathways of interest include disease (26 genes), signal transduction (32 genes), and cell–cell communication (four genes) (Table S4). The remaining 164 genes either did not have Ensembl identifiers or could not be characterised into pathways. However, inspection of GO terms suggests that a further 13 genes could be involved in immune system pathways (Figure 2, Table S5).

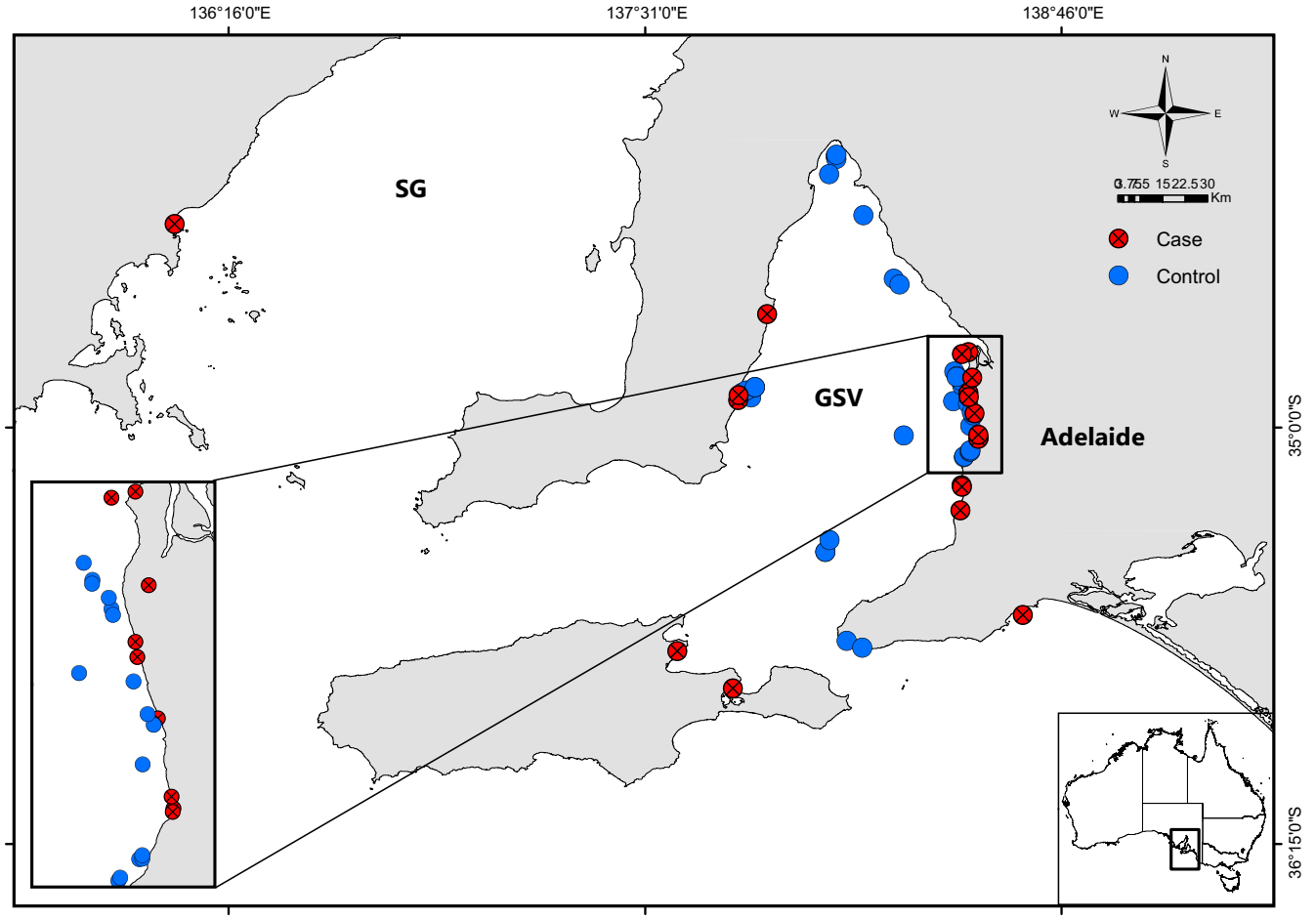


FIGURE 1 Sampling locations of Indo-Pacific bottlenose dolphins, *Tursiops aduncus*. Case (n = 19) are nonsurvivors and control (n = 34) are survivors from the 2013, Gulf St. Vincent outbreak used in the whole-genome association study of resistance and susceptibility to CeMV

Within the immune system pathways, genes were characterised into the innate, adaptive and cytokine signalling pathways. Nine genes (*PDIA3*, *FBXW10*, *FBXL7*, *UBA5*, *SEC31A*, *AREL1*, *LMO7*, *IKBKB* and *ASB11*) grouped into the MHC class I pathway of the adaptive immune system, which is important for recognising and fighting intracellular pathogens. Fc receptor proteins (FcRs) were also well characterised with ten genes (*DOCK1*, *MHY9*, *ACTR3*, *GRB2*, *NFATC2*, *CARD11*, *RASGRP2*, *IKBKB*, *MAPK8* and *CALM1*), while several F-box proteins were also identified (*LMO7*, *FBXL7*, *FBX10* and *FBXW11*). The *FBXW11* was identified through the candidate gene approach, as it had been previously proposed to be involved in CeMV susceptibility and resistance (Batley et al., 2019). Likewise, *MAPK8* was also identified as a candidate gene in this study (as well as in Batley et al., 2019), while the MAPK cascade was well characterised with four candidate genes (*FGF2*, *GRB2*, *BTC* and *CALM1*) and a further nine genes with GO terms relating to the MAPK cascades (*PDE6H*, *NTRK2*, *KIDINS220*, *INHBA*, *NPSH1*, *PLCE1*, *HRH4*, *TGFB3*, *IKBKB*). Finally, pathways and GO terms highlighted the importance of the regulation and expression of interleukins and T cells (see Table S4 for all pathways and subpathways).

Further inspection of the annotated SNPs and the gene regions they fall within revealed that majority of the SNPs fell within introns (n = 485). In total, 59 SNPs were found in exonic regions, in which 29 caused a missense mutation and 30 SNPs resulted in synonymous substitutions (Table S6). Of the SNPs that annotated to immune genes, twelve SNPs were found within exons; however, just six of these within three genes (*CD300LF*, *NFATC2* and *NFKBIZ*) caused a missense change, while six SNPs within four genes resulted in no amino acid change (*DOCK1*, *FBXW10*, *MASP1*, *MHY9*, *HRH4*, *KCTD5*) (Figure 2). The odds ratio (OR) suggest that for the SNPs that caused a missense change, the minor allele increased the odds of succumbing to CeMV (Figure 2). Other genes of interest that were annotated include *IL4α*, which had an OR of 16.25 (Figure 2) and *PATJ*, both of which have previously been proposed as potentially being involved in immune responses to morbilliviruses (Batley et al., 2019; Haralambieva et al., 2015; McCarthy et al., 2011).

3.3 | Candidate immune genes

At least 29 genes have been proposed to potentially play a role in immune responses to morbilliviruses in general (Hashiguchi et al., 2011; Haralambieva et al., 2015; McCarthy et al., 2011; Stejskalova et al., 2017), but only three of these (*PATJ*, *MAPK8* and *IL4α*) were found to

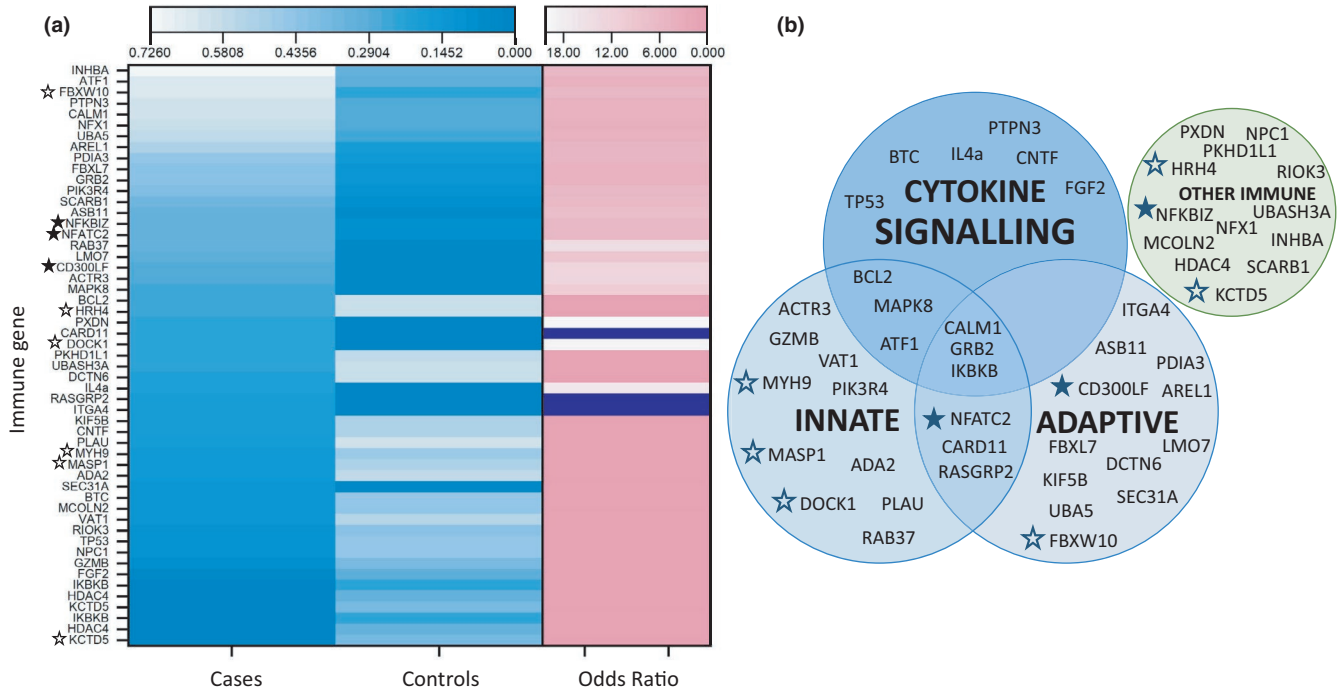
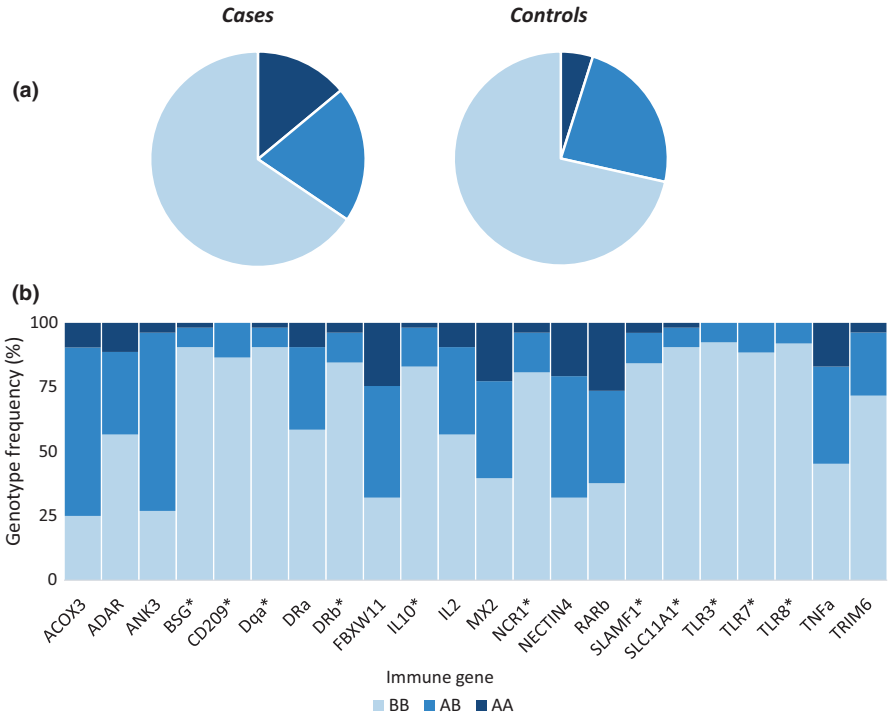


FIGURE 2 Thirty-six immune-related genes putatively associated with cetacean morbillivirus resistance and susceptibility. (a) Allele frequency differences between case and control individuals and their corresponding odds ratio. Blue odds ratios represent nonapplicable odds ratios (as allele frequency in controls =0). (b) Immune subpathways of the candidate immune-related genes. Other immune refers to the genes that had gene ontology (GO) terms relating to immune functions. Stars represent the seven genes that include exonic SNPs (filled, missense change; nonfilled, synonymous change)

FIGURE 3 Genotype distribution for the most differentiated SNP (χ^2) between case and control bottlenose dolphins for 23 genes previously suggested to be involved in morbillivirus immune responses. (a) Average genotype frequency for all 23 immune genes in cases and controls. (b) Genotype frequencies for each immune gene across all samples (cases and controls). *Denotes the genes with exceptionally high homozygosity (>84% of individuals)



be under putative selection between cases and controls in this study. For the remaining 26 genes, 22 aligned to the SABD reference genome. Within the aligned genes, 7,041 SNPs were extracted, of which 26 SNPs on four genes (*RARb*, *FBXW11*, *ANK3* and *ACOX3*) showed significant allele frequency differences ($p < .001$) between cases and controls. These SNPs were identified as outliers in the tests for selection but did not align to the common bottlenose dolphin proteins, and are therefore considered to be located within intronic, promoter,

or enhancer regions of the genes. The majority of the 22 genes were highly polymorphic (Table S7); however, inspection of genotype counts for the top performing SNP within each gene (i.e., SNP with greatest allele frequency differences between cases and controls) highlighted a lack of heterozygosity within 11 of the immune genes across all samples (Figure 3, Table S7). For these 11 SNPs, at least 84% of all samples were homozygotes. For *TLR8* and *TLR3*, all case samples were homozygotes, while only four control samples were heterozygotes. The genes *DQα*, *BSG* and *SLC11A1* also showed low levels of variation, with only four samples being heterozygotes.

4 | DISCUSSION

Host genetic factors play an important role in mobilising immune responses to invading pathogens, and may influence the outcome of an individual; yet relatively few studies have assessed the importance of these factors and immunogenetic diversity in wildlife disease risk (Bossart et al., 2019; Smith et al., 2009). Here, we used whole genome data sets to characterise genomic regions underlying resistance and susceptibility of dolphins to a highly contagious and fatal virus, CeMV. First, we provide additional support for previously proposed genes suggested to be associated with morbilliviruses and immune responses in general. This includes genes that were found to be under selection between case and control individuals from the same population using a RRS approach (Batley et al., 2019). We further expand on this, by uncovering host genetic variants across the entire dolphin genome, and in genes and pathways associated with immune functioning, including in MHC class I pathways involved in recognising pathogens. We also identified a lack of immunogenetic diversity in the studied dolphin population within immune-related genes previously recognised as important in the fight against pathogens in general. CeMV is of growing concern given ongoing climate change threatening to lead to more stressful environments for populations and species, potentially leading to immune suppression, and altering host and virus distributions (Burge et al., 2014). Since its discovery, CeMV has been reported to be the causative agent of several unusual mortality events across multiple cetacean species and populations (Di Guardo et al., 2005; Van Bressem et al.,1999, 2014). Through the identification of genes potentially involved in CeMV immune responses, our work clarifies how host genetic factors drive CeMV outcomes and provides knowledge about the diversity of immune responses, their interactions, and pathways in dolphins. More broadly, this work provides an example of how advancing technologies can enable greater insights into the role of host genetic factors in the variation of a trait across the entire genome, while also providing support for RRS approaches in conservation genomics.

4.1 | Comparison between RRS and WGS for identifying genes associated with disease resistance

As genomic technologies and capabilities continue to advance, conservation genomic techniques including reduced-representation and whole genome sequencing to assess variation of a trait will become more popular for wildlife populations. While the focus of this study is to understand host genetics variants in CeMV resistance and susceptibility, where possible it is important to compare and validate the potential use and constraints of RRS and whole genome sequencing approaches for addressing important conservation questions (Wright et al., 2020).

In this study, we increased the number of loci from 35,493 SNPs (Batley et al., 2019) to 7,720,686 SNPs across the genome to investigate associations between CeMV resistance and susceptibility and host genetic factors. In concordance with the RRS data set, estimates of inbreeding were not elevated in case samples compared to controls at the whole genome level, supporting previous suggestions that the outcome of an individual during this mortality event was not influenced by genome-wide inbreeding. Levels of inbreeding within wildlife populations have been associated with disease emergence, immunocompetence and increased disease susceptibility and severity (Smith et al., 2009; Valsecchi et al., 2004). While the GSV bottlenose dolphin population exhibits relatively low genetic diversity (Pratt et al., 2018), inbreeding estimates from both RRS and WGS did not suggest significant levels of inbreeding. This provides support for a lower density of SNPs (RRS) being sufficient for estimating inbreeding in this population.

In the case of the Tasmanian devil (*Sarcophilus harrisii*), which exhibits remarkably low genetic diversity, SNP density was too low in a RRS data set (>9,000 SNPs) to conduct a robust association study to identify genes associated with breeding success (Wright et al., 2020). While the SNP density was far greater for the bottlenose dolphin RRS data set of Batley et al. (2019), it was limited in its coverage across the genome, covering approximately 1% of its genome. This means that in addition to genes potentially not being represented in the RRS data set, gene regions may have been missed (e.g., exons). Despite this limitation, variation within introns of five candidate genes (*PATJ* [*INADL*], *MAPK8*, *FBXW11*, *ANK3* and *ACOX3*) between cases and controls were identified as potentially important genes for CeMV susceptibility. While these results are informative and important for understanding disease susceptibility in this population, complex traits are often determined by many genes with small effect (Santure & Garant, 2018), and the RRS approach to investigate a genotype-phenotype relationship may restrict the potential to identify variation in a large number of genes. The whole genome data set included a much larger SNP data set, and analysis identified significant differentiation between cases and controls in the same five genes as Batley et al. (2019), and in an additional 294 protein coding genes and uncharacterised proteins, with variation in at least 50 immune-related genes observed. Unlike the RRS approach, in this study variation was observed within exonic regions of the genome and SNPs within three immune genes (*CD300LF*, *NFATC2* and *NFKBIZ*) caused missense mutations. RRS data sets remain extremely important in conservation genomics, particularly when it is not feasible to sequence whole genomes, or when reference genomes are not available. For example, RRS was informative in elucidating the negligible influence of inbreeding on CeMV susceptibility and provided

a stride towards understanding the role of host genetic factors in CeMV immune responses. However, our comparison highlights the more comprehensive functional knowledge gained through a whole genome analysis.

4.2 | Gene functions and immune system pathways

The immune system plays a key part in the outcome of an individual and we therefore focus on pathways and genes associated to immune functions, however, other pathways and gene functions were also disclosed (see Table S3 and S4 for details). A wide range of well characterised immune related pathways were found to be putatively under selection, including a similar number of genes from both the innate and adaptive immune systems, as well as cytokine signalling pathways. These pathways are distinct, but interconnected (Gelain & Bonsembiante, 2019), reflecting the highly complex interactions and networks of the mammalian immune system.

4.2.1 | Innate immune system

The innate immune system is the first line of host defence and is rapid and nonspecific in its response to pathogens, involving the interplay of the complement system, pattern recognition receptors, cytokines and a diverse range of immune cells that detect and remove pathogens (Gui et al., 2013; Ohishi et al., 2011). A comparison between healthy and CeMV seropositive common bottlenose dolphins from estuaries in Florida and South Carolina revealed an upregulation of the innate immune system in seropositive dolphins, and in particular in lysozyme activity and monocytic phagocytosis (Bossart et al., 2019). In this study, the innate immune system was well characterised, with 19 genes found to be under selection between cases and controls. In particular, 10 genes were grouped into Fc receptor proteins (FcRs) that have important functions in the activation and downregulation of immune responses through their ability to bind to antibodies and stimulate cellular and humoral immune responses (Takai, 2002, 2005). Genes within this pathway (*DOCK1*, *MHY9*, *ACTR3*, *GRB2*) may be important for recognising foreign pathogens and stimulating phagocytosis to engulf and eliminate infectious agents (Acevedo-Whitehouse & Cunningham, 2006), while an additional three genes (*NFATC2*, *CARD11*, *CALM1*) may be important for the release of inflammatory mediators (Turner & Kinet, 1999). In humans, measles virus proteins have been reported to interact with FcRs to generate immunosuppression through impairment of cell function, decreased production of interleukins, and the loss of antigen specific T cell proliferation (Marie et al., 2001). Of particular interest, the gene *NFATC2* is part of a family that appear to be key mediators of immune responses, specifically by regulating the transcription of cytokine genes (*TNF-a* and *IL-13*) (Fric et al., 2014; Klein et al., 2006; Turner et al., 1998). These cytokine genes have been associated with defence against the measles virus (Haralambieva et al., 2015), and also may have been important in fighting infection in this population. While these genes (*TNF-a* and interleukins) are known to be important for morbilliviruses host defence, this finding highlights the need to look beyond cytokine receptors, at activators and initiators of these proteins, as they may play a role in an individual's response and outcome.

Neutrophils are among some of the most common white blood cells that circulate in the human body, participating in the inflammatory responses by releasing cytotoxic proteins during degranulation (Lacy, 2006; Naegelen et al., 2015). Neutrophils participate in inflammatory responses by releasing cytotoxic proteins during degranulation (Lacy, 2006; Naegelen et al., 2015). The morbillivirus infected dolphins in this study had a high prevalence (e.g., 18 out of 24 nonsurvivors examined) of lymphoid depletion in the spleen and lymph nodes (Kemper et al., 2016). This develops immunodeficiency resulting in secondary infections including from bacteria, protozoa, parasites and fungi (Di Guardo & Mazzariol, 2016). The presence of neutrophils in morbillivirus infected dolphin is a sign of an acute inflammatory response against those pathogens (Diaz-Delgado et al., 2017, 2019; Duignan et al., 1992). Here, we found evidence of selection within five genes (*GZMB*, *PLAU*, *ADA2*, *RAB37* and *VAT1*) that are involved in neutrophil degranulation. These results suggest that variation within these genes may play an important role in the release of cytotoxic proteins during neutrophil degranulation and may be key contributors to the coordination of an inflammatory response against the secondary infectious pathogens that follow CeMV.

4.2.2 | Adaptive immune system

The adaptive immune system, also known as the specific and non-rapid system, is mediated by B and T lymphocytes, and recognises pathogens by high affinity receptors (Werling & Jungi, 2003). In regards to CeMV infection in cetaceans, Bossart et al. (2019) observed a reduced adaptive immune response in CeMV seropositive dolphins, with a reduction in T cell lymphocyte proliferation and in T helper cells. Here, we found significant differentiation between cases and controls in 15 genes that were characterised into the adaptive immune system. Of particular interest is the MHC class I pathway, which is generally involved in the fight against viruses. In this study nine candidate genes were characterised into this pathway (*PDIA3*, *FBXW10*, *FBXL7*, *UBA5*, *SEC31A*, *AREL1*, *LMO7*, *IKBKB* and *ASB11*). The MHC complex is of known immune importance, being involved in resistance and susceptibility to disease through antigen processing and presentation (Acevedo-Whitehouse & Cunningham, 2006; de Sa et al., 2019). Genes here were characterised into antigen processing of the MHC class I pathway (*LMO7*, *AREL1*, *FBXL7*, *FBXL10*, *UBA5*, *IKBKB* and *ASB11*) whereby foreign proteins are degraded into short peptides for presentation to the MHC class I system (Strehl et al., 2005). The genes *PDIA3* and *SEC31A* are involved in antigen presentation, folding, and loading of MHC class I receptors that coordinates the movement of high-affinity peptides to MHC class I molecules (Santos et al., 2007; Scholz & Tampe, 2009). In addition, three genes (*DCTN6*, *KIF5B* and *SEC31A*) were characterised into the

antigen presentation of the MHC Class II pathway and may be important for presenting antigens to T lymphocytes that initiate an immune response (Moreno-Santillan et al., 2016). Across vertebrates, the MHC complex is one of the most well studied immune-related regions. It has been implicated in responses to measles vaccination (Haralambieva et al., 2015), and suggested to be functionally important in CeMV infection (Stejskalova et al., 2017). Although we found no evidence of selection within key MHC genes (e.g., *DQA*, *DQB*), variation in several downstream genes suggests that the MHC Class I and II pathways may be involved in a dolphin's ability to fight CeMV infection.

Some cell receptors may also play a role in modifying the response of immune cells. We found significant variation between cases and controls within four genes (*ITGA4*, *HRH4*, *IKBKB* and *CD300LF*) that may be involved in the regulation of immune functions. The gene *CD300LF*, found to contain four SNPs that cause a missense mutation may positively regulate the IL4-mediated signalling pathway by acting as a coreceptor for IL-4 (Moshkovits et al., 2015); a cytokine signalling gene that has been previously suggested to be important in morbillivirus immune responses, and found to be putatively under selection in this study. *ITGA4* may also promote viral resistance by permitting T lymphocytes to migrate to sites of inflammation.

A major immune response of humans to measles is controlled by T lymphocytes that recognise measles antigens (Haralambieva et al., 2015). These T lymphocytes also play a key role in immune responses of dolphins to CeMV, with seropositive dolphins showing a reduction in T cell proliferation in comparison to healthy dolphins (Bossart et al., 2011, 2019; Diaz-Delgado et al., 2019). Throughout the 2016 outbreak in GSV, all stranded dolphins showed clinical signs of lymphoid depletion (Kemper et al., 2016), suggesting that T cell proliferation may have been reduced, hampering the ability of an individual to fight the infection. Seven genes (*CARD11*, *NFATC2*, *GRB2*, *NFKBIZ*, *HDAC4*, *UBASH3A* and *IKBKB*) were found to be putatively under selection and may be involved in other adaptive pathways that relate to the signalling and differentiation of T and B cells. The candidate gene *NFKBIZ*, which contained a SNP that caused a missense mutation, may be of functional importance in the T cell receptor signalling pathway and in the regulation of inflammatory responses.

4.2.3 | Cytokine signalling in the immune system

Cytokines and their receptors are very important in the modulation of immune responses and are key components of host defence. Given their role in combating pathogens, cytokines have been the focus of several vaccination efforts against measles (Haralambieva et al., 2015), and were proposed as candidate genes for morbillivirus resistance and susceptibility (McCarthy et al., 2011). One of these cytokine signalling receptor genes, *IL4a*, was found to be under putative selection in our study, and selection in a further 11 genes were also characterised into this system (*CNTF*, *BCL2*, *CALM1*, *GRB2*, *ATF1*, *BTC*, *PTPN3*, *IKBKB*, *TP53*, *MAPK8* and *FGF2*). These findings provide further support that variation within cytokine signalling genes such as interleukins, and particularly *IL4a*, play an important role in host immune responses to morbilliviruses in general.

4.3 | Other pathways

Other candidate genes found under putative selection in dolphins are involved in multiple pathways that may indirectly be linked to immune responses. For example, signal transduction is an important process where extracellular signals, such as hormones or growth factors change the cell state or activity (Nair et al., 2019). Here, three candidate genes (*FGF2*, *GRB2*, *BTC* and *CALM1*) are involved in the MAPK family signalling pathway, and another nine (*PDE6H*, *NTRK2*, *KIDINS220*, *INHBA*, *NPSH1*, *PLCE1*, *HRH4*, *TGFB3*, *IKBKB*) were associated to the MAPK cascades. These are involved in the initiation of the innate immune system, activation of the adaptive immune system, and cell death after infection (Dong et al., 2002). *MAPK8* was previously suggested to be involved in dolphin susceptibility and resistance to CeMV, and was related to a response to heat stress (Batley et al., 2019). Likewise, it was found to be under selection in this whole genome study. Two other genes (*INHBA* and *BMPR1B*) are involved in signalling by the transforming growth factor family members that have important functions in the regulation of inflammatory responses and in T cell regulation and differentiation (Li et al., 2006). Transforming growth factors have been associated with immune responses to a range of diseases (Akdis et al., 2016), but to the best of our knowledge, they have not been implicated in immune responses to morbilliviruses.

4.4 | Candidate immune genes

Numerous genes have been proposed to be involved in immune responses to morbilliviruses, including binding genes, pathogen-associated molecular pattern sensing genes, cytokine-cytokine receptor genes, antiviral genes, and vitamin A and D receptor genes (Haralambieva et al., 2015; McCarthy et al., 2011; Stejskalova et al., 2017). Due to the high number of gene annotations, we mainly focused on SNPs that annotated to protein coding regions and therefore may have missed variation between cases and controls in intronic regions of important immune genes. We therefore assessed genetic variation and investigated putative signatures of selection in 22 genes previously proposed to be important in resistance and susceptibility to morbilliviruses. We found significant differentiation in intronic regions in four of such candidate genes (*RARb*, *FBXW11*, *ANK3* and *ACOX3)*. While the identified SNPs are within introns, the potential role of these genes in fighting CeMV should not be discarded. A large proportion of the mammalian genome is made up of introns (Chorev et al., 2017), and in the SABD reference genome less than 0.6% of SNPs are within exons (K.B. Batley, J. Sandoval-Castillo, L.B. Beheregaray, L.M. Möller, unpublished data). While many introns act in a neutral manner with

apparently no function, intronic SNPs might indirectly influence gene function and immune response genes through the alteration of splicing (Dhiman et al., 2008; Guigó & Ullrich, 2020; Seoighe & Korir, 2011; Singh et al., 2018).

Genetic diversity is essential for natural populations to adapt to rapid and ongoing changes to their environment (Manlik et al., 2019). Maintaining genetic diversity is particularly important for populations to recognise and fight infectious diseases (Hendricks et al., 2017). Immune genes are considered amongst some of the most polymorphic genes in wildlife populations (Dooley et al., 2018; Morris et al., 2015; Ruan et al., 2016), with diversity suggested to be maintained through pathogen-host balancing selection (Morris et al., 2015), and an excess of homozygous alleles probably impairing an individual's ability to successfully fight pathogens (Blanchong et al., 2016; Shafer et al., 2012; Smith et al., 2009). The dolphin population studied here has relatively low levels of standing genetic variation compared to neighbouring populations (Pratt et al., 2018; Pratt et al., in preparation), and this may have negatively influenced their susceptibility to CeMV. While we observed a high level of polymorphism in many immune genes, we found a lack of heterozygosity in some that are thought to be functionally important. This lack of diversity was observed across case and control samples, and therefore may not have led to case dolphins being more likely to succumb to CeMV, but the population being more susceptible. Within the GSV, Indo-Pacific bottlenose dolphins and common dolphins are considered resident species (Kemper et al., 2008). Common dolphins are very gregarious and form a larger population (Zanardo et al., 2016) and although a small number of common dolphin cases were recorded during the outbreak, the virus did not seem to have a similar impact in this population (Kemper et al., 2016). Common dolphins from GSV are more genetically diverse than Indo-Pacific bottlenose dolphins from the same bioregion (Barceló et al., 2021; Bilgmann et al., 2014; Pratt et al., 2018), and this difference in diversity may have influenced their ability to fight and survive CeMV infection.

5 | CONCLUSIONS

This whole genome association study disclosed the importance of key immune response genes and pathways in susceptibility and resistance of dolphins to the highly infectious and fatal CeMV. While the RRS study provided an important first step in uncovering genes potentially involved in CeMV immune responses, by expanding to a whole genome level, we have uncovered novel genes and pathways that have not previously been the target of morbillivirus immune response studies. In particular, the genes *CD300LF*, *NFATC2* and *NFKBIZ* may be involved in the regulation and expression of interleukins and T cells, while the gene pathways FcRs and MAPK cascade may be important for recognising pathogens and activating immune responses, and the initiation and activation of the immune system, respectively. In addition, we found evidence for putative selection in genes previously suggested to be potentially involved in responses to morbilliviruses, adding evidence that knowledge gained on immune responses by one species can be more broadly applied to other morbilliviruses. The results highlighted the importance of cytokines, T cells (particularly Th2) and *IL4*, in fighting infection by these viruses. Overall, our work highlights the complex interactions between the innate, adaptive, and signalling processes of the mammalian immune system in fighting infection by viruses and adds to our understanding of major marine mammal immune responses. The unravelled interactions of the immune systems emphasise the significance of whole genome studies to characterise the interplay of immune responses and genes involved in combating infections. Additional whole genome studies of larger CeMV outbreaks should clarify the role of these genes and pathways across virus strains, and cetacean populations and species.

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AUTHOR CONTRIBUTIONS

The study was designed by K.C.B. and L.M.M., in conjunction with L.B.B. Tissue samples from stranded dolphins and information about samples and the outbreak were provided by C.K. and I.T. Biopsy samples of live dolphins were collected by L.M.M. and N.Z. Laboratory work and bioinformatics were primarily conducted by Kimberley C. Batley, with guidance and assistance from J.S.-C. Data analysis and interpretation was conducted by K.C.B., with guidance from L.M.M., J.S.-C. and L.B.B., K.C.B. and L.M.M. wrote the paper, with critical revisions made by L.B.B, J.S.C., C.K., I.T. and N.Z.

DATA AVAILABILITY STATEMENT

The sequence data is available at NCBI's Sequence Read Archive (SRA), BioProject: PRJNA705721.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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