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Adaptive changes in the genomes of wild rabbits after 16 years of viral epidemics

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Abstract

Since its introduction to control overabundant invasive European rabbits (*Oryctolagus cuniculus*), the highly virulent rabbit haemorrhagic disease virus (RHDV) has caused regular annual disease outbreaks in Australian rabbit populations. Although initially reducing rabbit abundance by 60%, continent-wide, experimental evidence has since indicated increased genetic resistance in wild rabbits that have experienced RHDV-driven selection. To identify genetic adaptations, which explain the increased resistance to this biocontrol virus, we investigated genome-wide SNP (single nucleotide polymorphism) allele frequency changes in a South Australian rabbit population that was sampled in 1996 (pre-RHD genomes) and after 16 years of RHDV outbreaks. We identified several SNPs with changed allele frequencies within or close to genes potentially important for increased RHD resistance. The identified genes are known to be involved in virus infections and immune reactions or had previously been identified as being differentially expressed in healthy versus acutely RHDV-infected rabbits. Furthermore, we show in a simulation study that the allele/genotype frequency changes cannot be explained by drift alone and that several candidate genes had also been identified as being associated with surviving RHD in a different Australian rabbit population. Our unique data set allowed us to identify candidate genes for RHDV resistance that have evolved under natural conditions, and over a time span that would not have been feasible in an experimental setting. Moreover, it provides a rare example of host genetic adaptations to virus-driven selection in response to a suddenly emerging infectious disease.

KEYWORDS

adaptation, host–pathogen co-evolution, natural selection, rabbit, rabbit haemorrhagic disease virus, virus-driven selection

1 | INTRODUCTION

How hosts and their pathogens adapt to each other in different habitats, and how this shapes their genomes, is a fundamental question in evolutionary biology. Antagonistic adaptations and counter-adaptations between hosts and pathogens are important drivers of molecular evolution (Paterson et al., 2010). These processes have been revealed in experimental studies under laboratory conditions, in quickly evolving host–pathogen systems. For example, reciprocal adaptations (e.g. changes in virus pathogenicity and host resistance) in the nematode *Caenorhabditis elegans* and its microbial parasite *Bacillus thuringiensis* have been demonstrated (Schulte, Makus, Hasert, Michiels, & Schulenburg, 2010). Martins et al. (2014) reported genomic adaptations of *Drosophila melanogaster* in response to Drosophila C virus selection, and there are several more experimental studies demonstrating that insect hosts are adapting to different pathogens (reviewed by Kerstes & Martin, 2014). However, in complex natural environments many other factors simultaneously affect these processes. These include not only the host and pathogen population structures, genetic background, seasonal dynamics, reproductive rates and dependence on vectors, but also environmental and abiotic factors (reviewed by Maizels & Nussey, 2013; Pedersen & Babayan, 2011). Consequently, co-evolutionary adaptations are often more difficult to detect in nature. Yet, for fully understanding natural host–pathogen co-evolution, it is important to study such processes in the environments where they occur.

Rare opportunities for investigating these mechanisms under natural conditions are available through emerging infectious diseases (e.g. transmissible cancer in Tasmanian devils, Epstein et al., 2016; Hubert, Zerjal, & Hospital, 2018) or through pest biological control programmes, hereafter referred to as "biocontrol," (Di Giallonardo & Holmes, 2015). In classical biocontrol, a new predator, pathogen or parasite is released to reduce numbers of their host species, usually invasive species that became overly abundant in their new environment and are threatening native biodiversity and/or agricultural production. Like many emerging infectious diseases, successful biocontrol agents are, at the time of their release, highly virulent and cause high mortality in their host population. Nonetheless, the efficacy of the biocontrol agents often decreases due to the subsequent, co-evolutionary adaptive processes (reviewed by Goldson et al., 2014). These adaptations are believed to be the result of a reciprocal arms race in which pathogen virulence selects for improved host defence mechanisms to "escape," and vice versa (Daugherty & Malik, 2012; Dawkins & Krebs, 1979). Depending on the individual host–pathogen system, the efficacy of a biocontrol agent can change depending on the balance between virulence, transmission and host resistance.

There are numerous examples of such processes, mainly from biological control in plants or insect hosts, where the hosts developed resistance, or the biocontrol agent attenuated (reviewed by Hufbauer & Roderick, 2005). One well-known example of a biocontrol agent that quickly adapted to its mammalian host is the myxoma virus (MYXV). It was released in Australia in 1950 to control overabundant introduced European rabbits (*Oryctolagus cuniculus*). Only a few years later, different MYXV strains had evolved, the most successful variants in the field being strains with intermediate virulence, which produced high virus titres in skin lesions and allowed moderate host survival, thus insuring optimal transmission by biting insects (Fenner & Fantini, 1999).

In the years 1995–1996, another biocontrol virus was introduced into Australia to reduce the increasing rabbit numbers. The rabbit haemorrhagic disease virus (RHDV, Lagovirus, Caliciviridae) is a small, single-stranded (+) RNA virus that emerged in Europe in the 1980s, but was absent from Australia until it was deliberately imported. RHDV is highly virulent and initially caused mortality rates of around 95%, both in laboratory trials and in the field (Cooke & Berman, 2000; Mutze, Cooke, & Alexander, 1998). Since then, it has caused regular disease outbreaks in Australian rabbit populations (Mutze et al., 2014; Wells et al., 2015, 2018). Rapid evolution of the RHD virus has been reported, with an exceptionally high rate of nucleotide change observed in Australia (Eden, Kovaliski, et al., 2015; Kovaliski et al., 2013). RHDV appears to have reached a different dynamic equilibrium than the myxoma virus, likely because transmission is improved through high virulence. Rabbit carcasses attract insects such as blowflies that can transport the virus between rabbit populations (Schwensow et al., 2014). Recent Australian field isolates caused shorter survival times and higher mortality in wild rabbits than the biocontrol strain from the initial release (Elsworth et al., 2014).

Although genetic changes in the RHD virus are becoming better understood, little is known of the co-evolving genetic adaptations in rabbits. Experimental challenges of domestic and Australian wild rabbits confirm that the wild rabbits are evolving genetic resistance, and this may be more rapid in rabbit populations where RHDV outbreaks are frequent (Elsworth, Kovaliski, & Cooke, 2012). Furthermore, in one wild rabbit population, using a genome-wide SNP approach, genes enabling survival from acute RHD (Schwensow, Detering, et al., 2017), and evidence for RHD-driven selection on class I genes of the major histocompatibility complex (Schwensow, Mazzoni, et al., 2017), have been identified. It was also known that different RHDV strains bind differentially to different histo-blood group antigens (HBGAs) which are expressed on the duodenal surface and trachea of rabbits and were suggested as possible binding factors for RHDV (Nyström et al., 2011; Ruvoën-Clouet, Ganière, Andrè-Fantaine, Blanchar, & Le Pendu, 2000). Allele frequency differences after RHD outbreaks have been observed in wild rabbit populations in France (Nyström et al., 2011).

Put together, such data indicate that rabbit resistance is likely to involve many different genes with diverse functions and would need a genome-wide approach to identify candidate loci.

For this study, we took advantage of the "natural experiment" provided by the release of RHDV in Australia. We aimed to investigate how natural selection may have altered rabbit genomes following its release and we chose "genotyping by sequencing" (GBS, Elshire et al., 2011), a genome-wide next-generation sequencing approach, to identify genes under selection. We compared allele

frequencies of single nucleotide polymorphisms (SNPs) in rabbits that had been sampled in South Australia before RHD imposed selection on their genomes, with allele frequencies in the same rabbit population sampled 16 years later. We searched further for evidence for relevant genes for RHDV resistance by performing a meta-analysis using the SNPs from the present study and those that were identified being associated with surviving acute RHD in a different South Australian rabbit population (Schwensow et al., 2014).

2 | MATERIAL AND METHODS

2.1 | Samples

Rabbit samples had been collected in 1996 from Gum Creek sheep station and abutting Flinders Ranges National Park in northern South Australia (Figure 1) by the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Canberra (CSIRO ethics approval 95/96-21), as part of a serological study to follow the epidemiology of RHDV. Samples taken included liver tissue for virus-capture ELISA (*N* = 59) to detect early stages of infection as well as eye lenses and body weight for age estimation (Cooke, Robinson, Merchant, Nardin, & Capucci, 2000). The tissues used here were from rabbits which were too young to be fatally affected during the initial outbreak (Robinson, So, Müller, Cooke, & Capucci, 2002) and those which had not yet been infected. They are termed the "pre-RHDV selection" group. In 2012, sixteen years after the first collection, more samples were collected by Primary Industries and Regions South Australia (PIRSA), Adelaide (PIRSA ethics approval 11/09). Rabbits (*N* = 53) were collected in the same way by shooting at night with a 0.22-calibre rifle from a vehicle equipped with a 100 W spotlight. The location of each rabbit shot was recorded by GPS (Garmin 72, Schaffhausen, Switzerland) to nearest 10 m. A liver sample was collected from each rabbit using a sterile scalpel and stored in a labelled 5-ml Sarstedt vial and frozen. All samples were stored at −18°C. The 53 rabbits sampled in 2012 (post-RHDV selection) had been subject to approximately 15–16 generations under RHDV selection as every rabbit that is recruited into the breeding population would have had been previously exposed to RHD, and RHDV outbreaks had occurred regularly every year (Mutze et al., 2014, and references therein).

2.2 | Laboratory methods

DNA was extracted using the Qiagen's Gentra Puregene Mouse Tail Kit (Hilden, Germany) according to the manufacturer's protocol. Sixteen barcodes with a length of 4–9 nucleotides were designed using the GBS BARCODE GENERATOR (v.2.0, Deena Bioinformatics). GBS libraries were constructed using the restriction enzyme *PstI* (New England Biolabs) following a published protocol (Elshire et al., 2011) with the adjustment that we initially constructed an individual library for each rabbit (*N* = 108). All individual libraries were quality

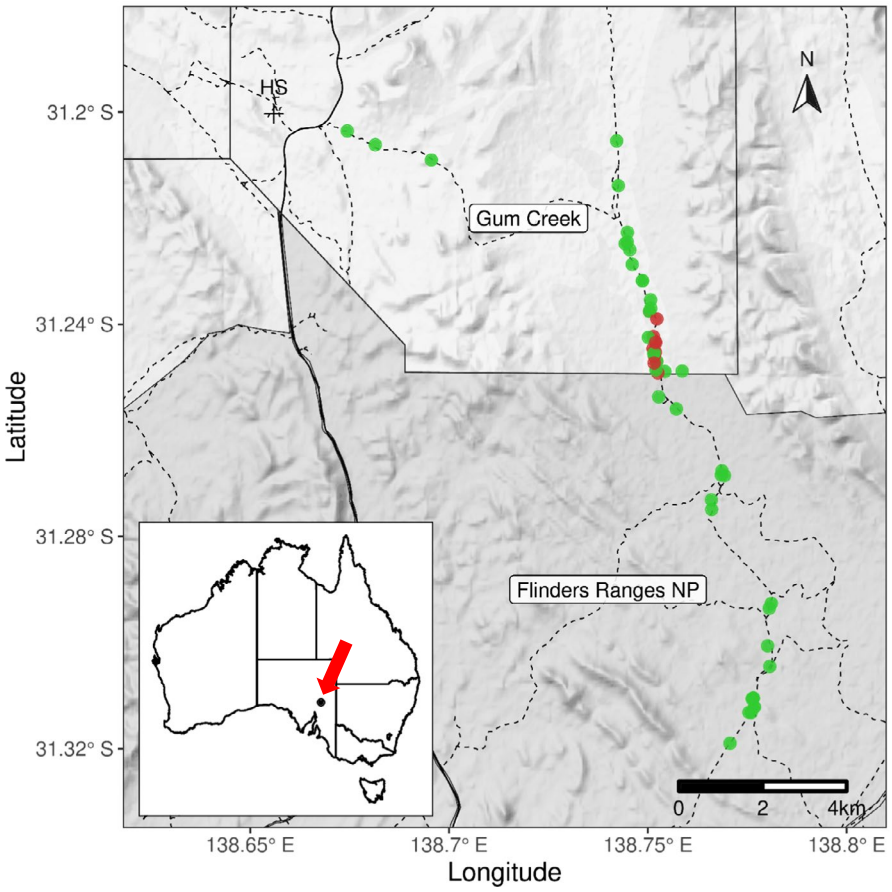

FIGURE 1 Map of the study site. Plotted are the GPS positions of the rabbits sampled in 2012. The positions coloured in red refer to the "inner" group of samples that were separated in a subsequent principal component analysis (Figure 2). The black dot indicated by a red arrow in the inset map shows the location of the Flinders Ranges within Australia. HS locates the Gum Creek station homestead [Colour figure can be viewed at wileyonlinelibrary.com]

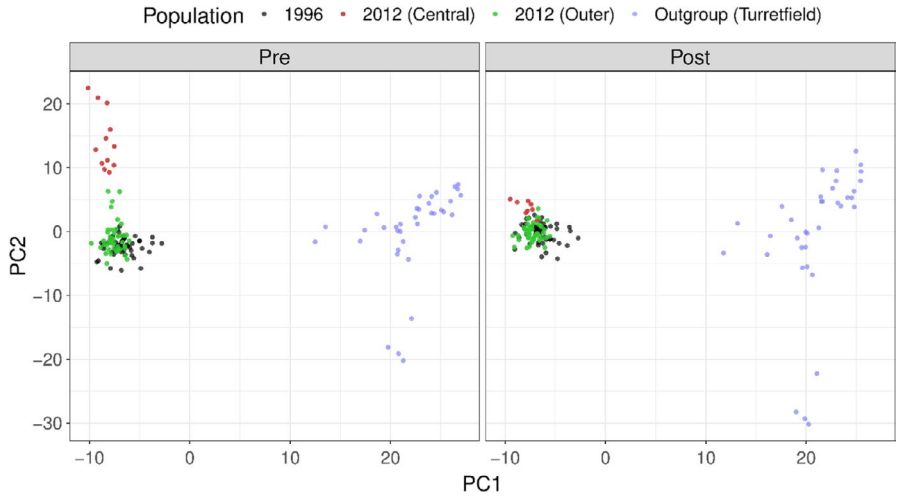

FIGURE 2 Plot showing the first two components of a principal component analysis (PCA), before (left panel "Pre") and after (right panel "Post") removal of SNPs which showed a distinct distributional pattern within the 2012 population. Samples from the original population in 1996 are coloured in black, while those from 2012 are coloured in red based on a k-means analysis on the prefiltered PCA which assigned them to the central collection region, and in green when taken elsewhere in the area; see also the map (Figure 1). Samples from the outgroup population in Turretfield, South Australia (ca. 400 km away, Schwensow, Detering, et al., 2017), are shown in blue [Colour figure can be viewed at wileyonlinelibrary.com]

controlled for fragment size distribution and absence of adapter dimers on an Agilent 2100 Bioanalyzer using the High Sensitivity Kit (Agilent). We prepared seven sequencing libraries by always pooling 16 individual libraries at equal molarities. Sequencing (paired-end 100-bp reads) was conducted on an Illumina HiSeq 2000 machine by the Beijing Genomic Institute (BGI), China.

2.3 | Sequencing data analysis

We performed standard quality assessment using FASTQC v.0.11.72 (http://bioinformatics.babraham.ac.uk/projects/fastqc/) and NGSREPORTS v1.1.2 (Ward, To, & Pederson, 2019). Initial libraries were trimmed using ADAPTER REMOVAL v2.2.1 (Lindgreen, 2012) using a quality threshold of 20 and discarding reads trimmed to <70 nt. Trimmed reads were demultiplexed using SABRE PE v1.0 (https://github.com/najoshi/sabre), incorporating the restriction site into the barcode and allowing for 1 mismatch. Restriction sites were additionally removed from R2 reads. Trimmed and filtered reads were aligned to v. 2.0 of the *Oryctolagus cuniculus* genome using BWA MEM v0.7.15 (Li & Durbin, 2009) with the default settings and the *-M* ("mark shorter split hits as secondary") option set, removing supplementary alignments and filtering on alignment qualities to only retain those with a score > 30 to ensure only the highest quality alignments were obtained. Thirty-seven additional samples collected in 2010 from a Turretfield population (Schwensow, Detering, et al., 2017) were also aligned and incorporated into subsequent SNP calling steps. Three samples in total (gc2700, gc2709 and pt1125) showed alignment rates < 35% and exhibited GC patterns unlike the remaining samples and were removed as poor quality samples. For SNP genotyping, the ref_map.pl pipeline of the STACKS suite v2.3b (Catchen, Amores, Hohenlohe, Cresko, & Postlethwait, 2011) was used using the default settings. The "populations" tool in STACKS suite v2.3b was then used to calculate basic population statistics, and only biallelic SNPs that were present in ≥75% of the individuals, and with a minor allele frequency >5%, were considered for subsequent stages of the analysis. We further excluded SNP alleles that were located on sex chromosomes or were not identified in the 1996 population. Five 1996 samples with a SNP identification rate < 50% were removed as outliers, which corresponded to samples with low recovery rates after demultiplexing, and unusual GC distributions. All variants were output as a VCF and converted to a GDS object for manipulation in R v3.6.2 (R Core Team, 2019) using the package SNPRELATE v.1.18.1 (Zheng et al., 2012). Linked SNPs were pruned using the snpgdsLDpruning function from SNPRelate setting a genotype correlation >40% as the threshold for linkage, and only taking the 1996 and 2012 samples into consideration.

2.4 | Population genetic analyses and outlier detection

Principal component analysis (PCA) was performed by using the pcrcomp function of the stats package in R (R Core Team, 2019) after removing samples with > 1/3 missing SNPs and by using only SNPs that were identified in > 95% of samples. For the purposes of PCA only, missing values were set to the minor allele frequencies across all populations. For assessing the magnitude of potential population structure and later use as an outgroup, we included GBS data from a recent study on rabbits sampled in 2010 in another South Australian rabbit population (Schwensow, Detering, et al., 2017) which is located approximately 400 km south of the population being studied here. The PCA revealed a strong similarity between 1996 and 2012 populations, but with distinct pattern within the 2012 population which correlated strongly with the collection region. As such, samples collected at this time point were

annotated as belonging to one of two collection regions (central or outer). SNP alleles were tested for any internal population structure using Fisher's exact test, and SNPs obtaining a raw *p*-value < .05 were not considered for comparison to the 1996 population. For the remaining candidate SNPs, analysis was performed using Fisher's test on genotype tables ("full genotype model") and allele frequency tables ("allele model"). The "full genotype model" assumes selection on certain genotypes (i.e. heterozygote or either homozygote), and the "allele model" assumes selection on specific alleles. We also used the FLK model (Bonhomme et al., 2010) on allele frequencies, as this model explicitly accounts for genetic drift. The FLK model requires a set of neutral loci and an outgroup for estimation of model parameters, and as such, the 2010 Turretfield population (Schwensow, Detering, et al., 2017) was used as an outgroup, with those >100kb from any annotated genes used as putative neutral loci. In addition to these analyses, a Bayesian approach as implemented in BayeScan (Foll & Gaggiotti, 2008) was used on the set of candidate SNPs. The sets of *p*-values returned by each of the genotype, allele and FLK analyses were adjusted to provide an estimate of the false discovery rate (FDR; Benjamini & Hochberg, 1995), and SNPs were selected as candidates for further study using a maximal FDR threshold of 0.05. A BayeScan *q*-value < 0.05 was also considered for these results; however, all SNPs with this value were detected by the previous two methods. We additionally estimated the effective population size (N_e) for both 1996 and 2012 using the single-sample linkage-disequilibrium method as implemented in NEESTIMATOR v2 (Do et al., 2014). Scripts for all analyses are available at https://uofabioinformaticshub.github.io/2014_SchwensowGBS/.

2.5 | Linkage analysis

Hill & Weir's equation (Hill & Weir, 1988) was used for linkage decay estimation by using a nonlinear fit and the 10,000 autosomal SNPs that were identified in> 95% of the individuals. Prior to genetic analyses (see above), SNPs had been pruned for linkage, so we also explored linkage of each identified significant candidate SNPs with any previously pruned SNPs within 40 kb. As phasing was not possible outside of a given STACKS locus, we used correlations between genotypes as a proxy for linkage. We calculated correlations between SNP pairs for each time point (1996 and 2012) separately as linkage may not be expected to be the same at each time point, given the selective bottleneck applied by RHDV. As these are single estimates of true linkage in each population, with many values obtained at the boundary points ($\rho = 1$), no formal analysis of changes in linkage could be performed and outliers were visually identified.

2.6 | Simulation of genetic drift

In order to assess the potential biological significance of putative SNPs, we simulated genetic drift in the absence of selective pressure to provide a baseline set of expectations. For this, we used a custom set of scripts collected as an R package and available from https://github.com/steveped/driftSim. As starting population parameters, we used our estimated effective population sizes N_e (see above) for 1996 and 2012, and one of the starting minor allele frequencies f0 = 0.05 to 0.5 increased in steps of 0.05. Each simulated rabbit in the initial population was then randomly assigned as either heterozygous or homozygous for the major or the minor SNP allele using f0. No selective advantage was specified for any allele. An initial survival rate was defined as $p = .1$, conservatively based on spotlight counts in the area after the first RHD outbreak (Mutze et al., 1998), with this functioning as an initial bottleneck and rabbits were assigned as survivors or fatalities with this probability. The initialization process was then repeated for either 4 or 8 neighbouring populations of the same size; however, we added variability to the initial allele frequencies on the logit scale using values $\sigma = 0.5$ and 0.8 to, respectively, represent similar or more divergent neighbouring populations. Considering the near-continuous nature of rabbit populations within vast areas of South Australia, we considered these to be conservative choices and likely overestimates. In order to express these values for σ in terms of correlations, we randomly sampled 1 million starting values for f0 (ranging from 0.05 through to 0.5) using the values of $\sigma = 0.5$ and 0.8 and these gave correlations of = 0.81 and 0.65, respectively. After simulation of the initial populations, we applied the same bottleneck procedure to each neighbouring population. During simulation of 16 generations, a migration rate of 0.15 was permitted along with an annual productivity of 20 young rabbits per breeding pair and a survival rate of 0.1. Five thousand simulations were performed for each initial f0 value, with 99.9% confidence bands generated for each starting value. For the purposes of plotting, smoothed values are shown using a polynomial fit as defined in the R package ggplot2 (Wickham, 2009).

2.7 | Gene Ontology (GO) enrichment analysis

Using Ensembl Release 96, we identified any gene within 40 kb of a SNP considered as significant and searched for potentially underlying functional connections by testing for enrichment of GO terms. We obtained the complete list of mappings from Ensembl gene IDs to GO terms using the R package BIOMART (Durinck, Spellman, Birney, & Huber, 2009). Using Fisher's exact tests, we then compared the frequency of GO terms of those genes within 40 kb of candidate SNPs (i.e. those with significantly different allele frequencies in 1996 and 2012) with GO term frequencies in all genes close to the remaining (i.e. nonsignificant) SNPs. We removed all GO terms which mapped to two or fewer genes and adjusted all p-values using Benjamini–Hochberg's FDR. GO terms with 3 or fewer steps back to the ontology root were additionally excluded to ensure more informative results.

2.8 | Meta-analysis

The results were then compared to the results of our earlier study that identified loci potentially important for surviving acute RHD

(Schwensow, Detering, et al., 2017) in a different rabbit population ca. 400 km away from the current study site. For this meta-analysis, we used Fisher's method (Fisher, 1925) to combine p-values from both genotype analyses, and resultant *p*-values were drawn from a chi-square distribution with 4 degrees of freedom (i.e. 2*k*). Bonferroni's adjustment was used to control the family-wise error rate (FWER) at 0.05, with a more generous threshold also applied using Benjamini and Hochberg's FDR.

3 | RESULTS

3.1 | Sequencing results, genome coverage and linkage decay

We obtained a total of 486,524,954 100 nt paired-end raw reads across the seven Illumina sequencing lanes. After demultiplexing, reads recovered for each sample ranged between 1,042,523 and 9,659,406, with an average of 4,003,924 paired reads per sample. Of those, 82.5% could be mapped to the OryCun 2.0 reference genome. The mapping rates for some of the 1996 samples were slightly lower (mean = 82.1%, range: 65.7%–86.0%) than for the 2012 samples (mean = 82.9%, range: 74.1%–86.3%) which was not surprising given the age of these samples. After performing all subsequent SNP filtering steps (see Material & Methods), we retained 18,878 SNPs for the subsequent analyses, with a mean distance between SNPs of 139.25 kb. After removal of SNPs showing a collection region effect in the 2012 population, we used a final data set of 17,102 SNP loci.

The estimated distance at which linkage between SNPs falls to half of the maximum value was 57 kb (Figure S1), and a previous study had identified strong disease-associated signals at 40 kb distance to genes (Lehne et al., 2011). We chose a conservative approach and concentrated for the subsequent analysis on those 8,690 SNPs that were within 40 kb of 6,287 annotated genes (see Table S1 for SNP positions and their distance to genes). We used further 3,179 SNPs that were situated> 100 kb away from a known gene to generate a putatively neutral subset of loci for the estimation of the co-ancestry matrix for the FLK analysis.

3.2 | Population size and structure

Allowing for a lowest allele frequency of 0.5, the estimated effective population size for the 1996 population was N_e = 222 (95% CI: 221.2, 222.9) and N_e = 115.9 (95% CI: 115.7, 116.1) for the 2012 population. Data exploration using PCA revealed that rabbits sampled in 2012, from the centre of our collection region, were less similar to the 1996 population than those sampled at the edges of the sampled region (Figure 2). To avoid possible effects of stratification within the 2012 population, we used Fisher's exact tests to identify the 1,682 SNPs having a possible structural component and excluded those SNPs (not the individuals) from subsequent analysis. This ensured that our final data set was primarily testing for changes in allele distributions between 1996 and 2012 (Figure 2) and that internal structure within the 2012 population was not biasing results. All samples were clearly different to the outgroup from Turretfield, South Australia (data from Schwensow, Detering, et al., 2017), which is about 400 km south of the study population.

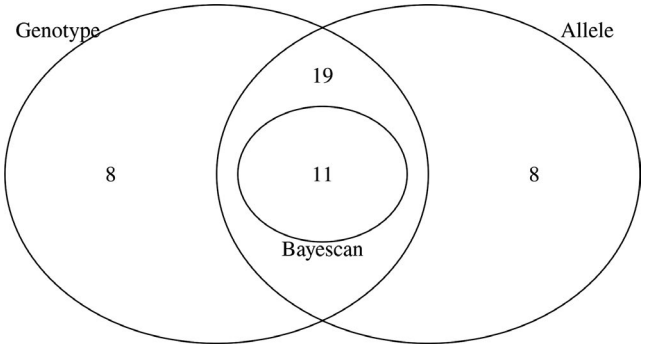

FIGURE 3 Venn diagram summarizing the number of SNPs identified as showing changes in allele or genotype frequencies between 1996 and 2012, using each of the three approaches. SNPs were considered as significant after applying a Benjamini–Hochberg FDR of 0.05. BayeScan results were considered as significant if the q-value was below 0.05

3.3 | Candidate SNP loci for RHDV-driven selection

Using the "full genotype model," we found 15 SNPs with significantly changed frequency applying FWER at α = 0.05. When applying an FDR of 0.05, the full genotype model found 38 SNPs with different genotype frequencies between 1996 and 2012 (Figure 3). For the most highly ranked SNP (3391201_92, Figure S2), the homozygote minor allele genotype had been completely lost in the 2012 population. A reduction in the minor allele frequency and shift towards the homozygous major allele was typically observed in the SNPs with particularly strong statistical support (i.e. with a Bonferroni-adjusted *p*-value < .05, Figure S2). The "allele model" identified five SNPs with changed allele frequencies applying FWER at α = 0.05 and 38 SNPs when controlling the FDR at α = 0.05. Five of the SNPs have also been identified by the FLK analysis but only after relaxing the FDR to α = 0.1 (data not shown). BayeScan identified 11 candidate SNPs with *q*-value < 0.05, all of which were also identified by the "full genotype model" and the "allele model" (Figure 3). In total, that gave 46 unique SNPs identified by one or more of the three models.

3.4 | Mapping the candidate SNPs to the rabbit genome

When we mapped the 46 SNPs identified with either model to known genes, we found that 32 of them were located within

TABLE 1 Genes that are in within 40 kb of the set of SNPs found as significantly different between 1996 and 2012 under the genotype model, the allele model, and by the BayeScan analysis. Shown are the location of the SNP on the chromosome or scaffold, respectively, the SNP ID, the Ensembl gene ID (gene ID), the gene name, the distance of the SNP to the closest genes (< 40 kb distance). The closest gene to a SNP is printed in bold. The model under which this SNP is given (A = "allele model"; G = "genotype model"; B= "BayeScan"). If a function/association with other virus infections or immune responses has been described or this gene has been found to be differentially expressed in acutely infected versus healthy rabbits, this is indicated as "function" and the respective reference is given. For more information, see supplementary file 1

SNP location (chromosome:position)	SNP ID	Gene ID	Gene name	Distance	Model	Function	References
1:64,635,286	**87917_119**	**ENSOCUG00000007758**	**CEP78**	**Intronic**	**AG**	**Downregulated in acute RHD, virus**	Hossain et al. (2018), Schwensow et al. (2012)
2:139,723,776	**482617_100**	**ENSOCUG00000001127**	**RHOQ**	**8,346**	**G**	**Virus**	Liang et al. (2017)
2:139,723,776	482617_100	ENSOCUG00000021835	ATP6V1E2	10,081	G		
2:139,723,776	482617_100	ENSOCUG00000028184	TMEM247	37,653	G		
3:56,309,981	**644062_1**	**ENSOCUG00000004827**	**SFXN1**	**Intronic**	**G**	**Meta-analysis**	
3:90,851,512	**686773_29**	**ENSOCUG00000009616**	**CRISPLD1**	**Exonic**	**AG**	**Immune response**	Gibbs et al. (2008)
4:35,111,855	**836950_151**	**ENSOCUG00000012144**	**TMPRSS12**	**5,745**	**ABG**	**Virus**	Yun et al. (2016)
4:89,602,973	**916731_91**	**ENSOCUG00000006126**	**RIC8B**	**Intronic**	**AG**	**Upregulated in acute rhd**	Schwensow et al. (2012)
6:9,037,073	**1009106_1**	**ENSOCUG00000027083**	**CLEC19A**	**15,180**	**ABG**		
7:2,702,919	**1045185_75**	**ENSOCUG00000027861**		**3,276**	**A**		
7:2,702,919	1045185_75	ENSOCUG00000007975	ZNF777	12,875	A	**Virus**	(PathCards, Belinky et al., 2015)
12:60,113,885	**1902686_98**	**ENSOCUG00000001871**		**12,820**	**AG**		
12:60,113,885	1902686_98	ENSOCUG00000016906		13,295	AG		
12:60,113,885	1902686_98	ENSOCUG00000021694		18,149	AG		
12:60,113,885	1902686_98	ENSOCUG00000023798		35,410	AG		
12:141,844,290	**1997286_93**	**ENSOCUG00000004829**	**ESR1**	**Intronic**	**A**	**Downregulated in acute RHD, virus**	Schwensow et al. (2012)
13:129,650,867	**2227006_109**	**ENSOCUG00000022823**	**ID3**	**16,426**	**ABG**	**Virus, apoptosis**	Menner et al. (2015), Rauch et al. (2017)
14:97,813,263	**2405359_66**	**ENSOCUG00000005835**	**STXBP5L**	**Intronic**	**A**	**Virus**	Zhang et al. (2016)
15:87,473,844	**2580149_42**	**ENSOCUG00000001910**	**ADGRL3**	**Intronic**	**AG**		
16:56,527,308	**2689075_49**	**ENSOCUG00000000984**	**ESRRG**	**Intronic**	**AG**		
18:25,311,176	**2906428_79**	**ENSOCUG00000011533**	**RHOBTB1**	**24**	**AG**	**Immune response**	Bokoch (2005)
19:19,178,148	**3016258_99**	**ENSOCUG00000015254**	**SEZ6**	**Intronic**	**AG**		
19:26,020,372	**3032107_92**	**ENSOCUG00000024664**	**LHX1**	**20,607**	**AG**	**Virus**	
19:26,020,372	3032107_92	ENSOCUG00000014434	AATF	30,904	AG	**Downregulated in acute RHD, apoptosis**	Schwensow et al. (2012)
19:30,689,426	**3040789_114**	**ENSOCUG00000010440**	**MSI2**	**Intronic**	**AG**	**Virus**	Wang et al. (2015)

(Continues)

TABLE 1 (Continued)

SNP location (chromosome:position)	SNP ID	Gene ID	Gene name	Distance	Model	Function	References
GL018704:2,271,135	**3378006_1**	**ENSOCUG00000006255**	**AGO3**	**9,610**	**G**	**Virus, meta-analysis**	Ferreira et al. (2018)
GL018704:2,271,135	3378006_1	ENSOCUG00000027798		16,486	G		
GL018704:2,271,135	3378006_1	ENSOCUG00000029722	TEKT2	20,166	G		
GL018704:2,271,135	3378006_1	ENSOCUG00000011038	ADPRHL2	24,425	G	**Meta-analysis**	
GL018704:2,271,135	3378006_1	ENSOCUG00000011042	COL8A2	34,195	G	**Meta-analysis**	
GL018705:1,937,359	**3391201_92**	**ENSOCUG00000016428**	**RCBTB2**	**37,689**	**ABG**		
GL018751:737,318	**3695415_108**	**ENSOCUG00000003672**	**TRAF3**	**Intronic**	**ABG**	**Antiviral immune response**	Zhu et al. (2019)
GL018751:737,318	3695415_108	ENSOCUG00000010323	CDC42BPB	5,698	ABG		
GL018791:957,960	**3829050_109**	**ENSOCUG00000007849**	**FHAD1**	**Intronic**	**AG**		
GL018883:283,912	**3999128_75**	**ENSOCUG00000016318**	**SERPINA6**	**Intronic**	**A**	**Virus, liver failure**	Sun et al. (2019)
GL018883:283,912	3999128_75	ENSOCUG00000016273	PPP4R4	21,034	A		
GL018907:283,766	**4010189_98**	**ENSOCUG00000008870**	**BRWD1**	**Intronic**	**ABG**	**Immune response**	Mandal et al. (2018)
GL018933:204,058	**4044777_119**	**ENSOCUG00000005859**	**FNBP1**	**Intronic**	**ABG**		
GL018985:123,328	**4098838_14**	**ENSOCUG00000006396**		**Intronic**	**A**		
GL018985:123,328	4098838_14	ENSOCUG00000006403	CLDN5	14,238	A	Virus, upregulated.	Schwensow et al. (2012)
GL018985:123,328	4098838_14	ENSOCUG00000025709	UFD1	28,184	A		
GL018985:129,495	**4098854_101**	**ENSOCUG00000006396**		**Intronic**	**AG**		
GL018985:129,495	4098854_101	ENSOCUG00000006403	CLDN5	20,405	AG	Virus, upregulated.	Schwensow et al. (2012)
GL018985:129,495	4098854_101	ENSOCUG00000025709	UFD1	22,017	AG		
GL019049:178,272	**4136275_162**	**ENSOCUG00000026644**		**Intronic**	**G**		
GL019049:178,272	4136275_162	ENSOCUG00000017433		11,078	G		
GL019049:178,272	4136275_162	ENSOCUG00000009045	ASS1	36,520	G		
GL019077:34,265	**4149102_107**	**ENSOCUG00000024268**	**IFT140**	**Intronic**	**AG**		
GL019077:34,265	4149102_107	ENSOCUG00000000521	TELO2	6,323	AG		
GL019077:34,265	4149102_107	ENSOCUG00000001136	TMEM204	13,406	AG	**Inflammation, pathological processes**	Kearsey, Petit, De Oliveira, and Schweighoffer (2004)
GL019077:34,265	4149102_107	ENSOCUG00000027299	PTX4	19,359	AG		
GL019084:74,038	**4146664_90**	**ENSOCUG00000010071**	**GNB1**	**Intronic**	**AG**	**Virus**	Lim et al. (2012)
GL019084:74,038	4146664_90	ENSOCUG00000021568	NADK	12,587	AG	**Cellular defence**	Lerner, Niere, Ludwig, and Ziegler (2001)
GL019084:74,038	4146664_90	ENSOCUG00000007721		30,580	AG		

(Continues)

40 kb of 57 genes; the remaining 14 SNPs were > 40 kb away from genes or on unplaced scaffolds. One of the SNPs (686773_29) was within the coding region of the gene CRISPLD1 (Table 1), while a further 19 were within intronic regions. All identified genes were considered as potential targets of RHDV-driven selection.

3.5 | Changes in linkage between 1996 and 2012

One pair of SNPs (2028473_73 and 2028554_103) at a distance of 38.1kb was visually identified as showing a potential change in linkage between sampling dates. While genotypes were 100% correlated in 1996, this dropped to 1.5% correlation in 2012. A further SNP at a distance of 74.8 kb from 2028473_73 showed a similar pattern dropping from 100% to a correlation of 13% in 2012. All other SNPs within this region were unlinked with the primary candidate SNP. The closest gene is the E3 ubiquitin–protein ligase COP1 but it is a further 40kb upstream from the most distal SNP to the primary candidate SNP.

3.6 | Genetic drift modelling

After simulation of genetic drift, the final allele frequency clearly remained distributed around the initial starting frequency. However, the variability of the final allele frequency was heavily affected both by the number of neighbouring subpopulations and their initial similarity to the population of interest. Assuming eight rather than four surrounding subpopulations tended to hold the allele frequencies in the study population steadier over time (Figure 4). A higher degree of variability between all populations resulted in wider distributions and more variable allele frequencies after allowing for drift. Simulations assuming eight neighbouring populations that were more similar to the population of interest provided results that resembled the observed changes in allele frequency most closely; however, under more conservative assumptions, several significant SNP loci were still found to be outside the simulated ranges. The simulations add further support that these particular loci were likely to have experienced selective pressure (rather than underlying drift alone) between 1996 and 2012.

3.7 | Gene ontology analysis

No significant enrichment was identified considering all genes up to a maximal distance of 40 kb. The most highly ranked GO terms corresponded to an FDR threshold of 21.6%. Of those, the terms of most interest with respect to RHDV-driven selection were “regulation of defence response to virus,” “pattern recognition receptor signalling pathway,” “intracellular receptor signalling pathway” and “single-stranded RNA binding” (data not shown).

TABLE 1 (Continued)

SNP location (chromosome:position)	SNP ID	Gene ID	Gene name	Distance	Model	Function	References
GL019154:883	**4176850_23**	**ENSOCUG00000002484**	**MTHFSD**	**828**	**AG**		
GL019154:883	4176850_23	ENSOCUG00000001701	FOXF1	19,026	AG	**Lung disorder**	Szafranski et al. (2013)
GL019274:63,350	**4208471_113**	**ENSOCUG00000015189**		**37,542**	**G**		

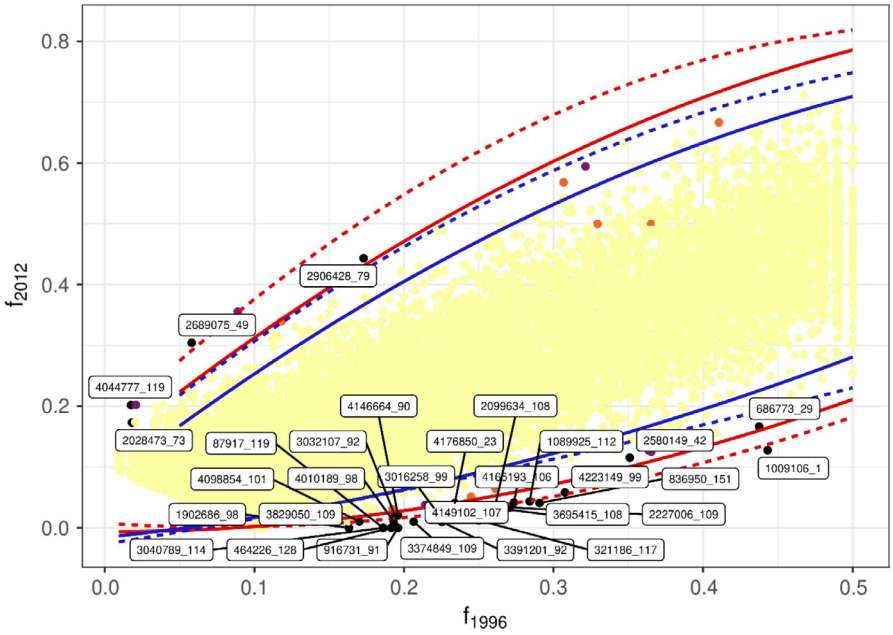

FIGURE 4 Comparison of the observed SNP allele frequencies in the 1996 and 2012 populations with the simulated prediction intervals assuming drift. SNPs considered as significant (FDR = 0.05) under the genotype model are highlighted (red = significant in the genotype model; purple = significant in the allele model; black = significant in both models), while nonsignificant SNPs are indicated by yellow dots. The 99.9% prediction intervals are indicated by the bands for differing simulation parameters. Intervals generated by 4 neighbouring populations are shown in red, while intervals generated by 8 neighbouring populations are shown in blue. Neighbouring populations simulated with the closest similarity to the main population are shown with solid lines ($\sigma = 0.5$), while those with less similarity are shown as dashed lines ($\sigma = 0.8$). The two points which clearly fall within all bands were found significant under the full genotype model and show differences in heterozygosity rather than in the allele frequencies themselves. The inner blue bands represent the expected scenario which presumably most closely resembles the ecological system under investigation, with a continuous population of rabbits (i.e. a large number of neighbouring populations), with highly correlated allele frequencies. Labels indicate IDs of those SNPs [Colour figure can be viewed at wileyonlinelibrary.com]

3.8 | Meta-analysis

Of the 9,229 unique SNPs earlier identified (Schwensow, Detering, et al., 2017), only 1,431 were in common with the data set derived from this current study. We identified two common SNPs from both studies using Bonferroni's adjustment to control the FWER at 0.05 and 10 SNPs when applying the more relaxed criterion of an FDR < 0.05. These 10 SNPs were located within or in close proximity to 22 genes (Table 2). Eight of the 10 SNPs were in proximity to at least one gene with known functions in virus infections or immune reactions, and four of the genes were previously found to be up- or downregulated in rabbits with acute RHD (Schwensow, Cooke, Fickel, Lutz, & Sommer, 2012).

4 | DISCUSSION

Direct measurements on genetic changes in mammalian hosts in response to selection through infectious diseases are rare. It was therefore exceptional that a collection of archived tissues allowed us to investigate natural selection on wild rabbits after the introduction of the biocontrol virus RHDV. From wild rabbits sampled in north-eastern South Australia in 1996 and again in 2012, we identified ~ 19k genome-wide SNPs using GBS, with 8,690 of them being in a maximal distance of 40 kb to 6,287 annotated rabbit genes. This approach differed markedly from previous studies which used candidate gene approaches and reported allele frequency changes after RHD outbreaks at genes important for synthesis of rabbit HBGA ligands (Guillon, Ruvoen-Clouet, Le Moullac-Vaidye, Marchandeau, & Le Pendu, 2009; Nyström et al., 2011) or different major histocompatibility complex (MHC) supertype frequencies in rabbits that survived RHD and those that died of it (Schwensow, Mazzoni, et al., 2017).

From samples taken 16 years apart, we identified genes potentially under RHD virus-driven selection by repeated annual outbreaks of disease. Expedient for this study was the availability of an annotated reference genome, allowing us to map our identified SNPs to genes. Biological knowledge about the functions of the identified genes could then be considered further, to assess whether the genes and mechanisms identified are indicative of RHDV-driven selection. We considered that any conclusion would be strengthened if the same genes were associated with other viral diseases or had been identified in previous studies, and we have reviewed the available literature accordingly and provide brief information on the functions of the identified candidate genes (Text S1).

The study identified 46 SNPs with allele/genotype frequencies that have demonstrably changed within a rabbit population during the 16 years following the first emergence of RHDV in this

TABLE 2 Significant genes the meta-analysis that compared the current candidate genes to the ones previously identified (Schwensow et. al 2017b) in a different rabbit population. Shown are the location of the SNP on the chromosome or scaffold, respectively, the SNP ID, the Ensembl gene ID (gene ID), the gene name and the estimate of the FDR. If a function/association with other virus infections or immune responses has been described or this gene has been found to be differentially expressed in acutely infected versus healthy rabbits, this is indicated as "function" and the respective reference is given. For more information, see supplementary file 1

SNP location (chromosome:position)	SNP ID	Gene ID	Gene name	FDR	Function	References
3:56309981	644062_1	ENSOCUG00000004827	SFXN1	0.002176		
GL018704:2271135	3378006_1	ENSOCUG00000011042	COL8A2	0.01535		
GL018704:2271135	3378006_1	ENSOCUG00000011038	ADPRHL2	0.01535		
GL018704:2271135	3378006_1	ENSOCUG00000029722	TEKT2	0.01535		
GL018704:2271135	3378006_1	ENSOCUG00000006255	AGO3	0.01535	virus	Ferreira et al. (2018), Schwensow et al. (2012)
GL018847:216450	3941015_20	ENSOCUG00000012298	ODF2	0.02311	paralogue regulated in acute RHD	Schwensow et al. (2012)
GL018847:216450	3941015_20	ENSOCUG00000008855	CERCAM	0.02311	hepatic inflammation, liver failure	Chen, Yan, Wang, Xu, and Deng (2014)
1:146274492	213788_4	ENSOCUG00000000568	HBB2	0.02477		
1:146274492	213788_4	ENSOCUG00000021180	HBG2	0.02477		
1:146274492	213788_4	ENSOCUG00000027533	HBG1	0.02477		
1:146274492	213788_4	ENSOCUG00000026912	OR51I1	0.02477	upregulated in acute RHD	Schwensow et al. (2012)
1:146274492	213788_4	ENSOCUG00000025404	OLFR64_1	0.02477		
20:14614986	3118382_68	ENSOCUG00000008913	RPS6KA5	0.02477	downregulated in acute RHD, inflammation	Deak et al. (1998), Schwensow et al. (2012)
20:10514947	3108287_38	ENSOCUG00000008859	KCNH5	0.03055		
21:6185579	3156991_0	ENSOCUG00000005961	KDM2B	0.03339		
21:6185579	3156991_0	ENSOCUG00000005957	RNF34	0.03339		
14:114833115	2425370_49	ENSOCUG00000011289	ALCAM	0.03394	virus infection	Park et al. (2017), Stachowiak and Weingartl (2012)
GL018828:225354	3904662_77	ENSOCUG00000027620	PAQR4	0.04325	tumour development	Zhang et al. (2018)
GL018828:225354	3904662_77	ENSOCUG00000017181	PKMYT1	0.04325	virus infection	Bryan, Dyson, and Akula (2006)
GL018828:225354	3904662_77	ENSOCUG00000027985	TNFRSF12A	0.04325	immune reactions	
GL018828:225354	3904662_77	ENSOCUG00000017197	CLDN6	0.04325	virus infection, downregulated in acute RHD	Schwensow et al. (2012)
GL018864:1306	3970912_82	ENSOCUG00000021601	SCARB1	0.04325	virus infection, upregulated in acute RHD	Schwensow et al. (2012)

population. Thirty-two of the SNPs were within 40 kb distance to 57 known genes. While 12 of these candidate genes have not been functionally characterized as yet, the majority have known functions in antiviral immune responses, apoptosis or other disease responses (Bokoch, 2005; Gibbs, Roelants, & O'Bryan, 2008; Zhu et al., 2019, see supplement for further information). Apoptosis of hepatocytes was found to be induced during RHD (Jung, Lee, Tai, Park, & Lee, 2000; Niedźwiedzka-Rystwej & Deptuła, 2012). In our previous study, we found an overrepresentation of apoptosis-related GO terms in the genes that had different allele frequencies between RHD-resistant rabbits and those that died of an infection suggesting that this mechanism is important for surviving RHDV infection (Schwensow, Detering, et al., 2017). Fourteen of the identified candidate genes, for example TMPRSS12, ID3 and MSI2 (Table 1), play a role in different virus infections (e.g. Menner et al., 2015; Rauch et al., 2017; Wang et al., 2015; Yun et al., 2016). The most highly ranked SNP (3391201_92) had lost its minor homozygote genotype completely in 2012, and most individuals were homozygous for the major allele. While this suggests strong selection against the minor allele, we cannot speculate about the functional background because this SNP is located on an unplaced scaffold and the currently closest mapped gene, RCBTB2, is ~37 kb from the SNP and encodes a poorly characterized protein.

We found that most changes in allele frequencies were probably due to factors beyond drift alone. Our simulation approach to model genetic drift in the absence of selective pressure gave 99.9% confidence bands for changes in allele frequency. Most SNPs with evidence of selection fall outside of this range. Only two SNPs, 3378006_1 (close to the genes COL8A2, ADPRHL2, TEKT2 and AGO3) and 644062_1 (close to gene SFXN1), fell clearly within all bands and both were unusual in that no significant change was observed in the allele frequencies. Rather both homozygous genotypes had been lost in the 2012 population such that only heterozygous individuals had been detected. As sequencing depth was not higher than usual, we do not assume that this observation is due to duplication. The implications of this are not immediately clear, but it is interesting to note that both SNPs were significant in our meta-analysis (see below).

Previously, we had also used genome-wide SNPs to compare allele frequency differences between RHD-susceptible rabbits that had acquired an RHDV infection and died to those that were fully susceptible and survived (Schwensow, Detering, et al., 2017). While we identified candidate genes that potentially enabled rabbits to survive RHD, that study did not necessarily reveal long-term adaptive changes over years of virus-driven selection. While we identified candidate genes that potentially enabled rabbits to survive RHD, that study did not necessarily reveal long-term adaptive changes over years of virus-driven selection. Therefore, we performed a meta-analysis comparing the genes identified on the basis that changed alleles or genotype frequencies in both studies would be further evidence for a role in RHD resistance. Surprisingly, we found that despite using the same method and restriction enzyme, only 1,431 SNPs of the > 9,000 SNPs identified in the earlier study were also found in the current study. We assume that several factors contribute to this low level of overlap. First, in the current study we have improved our demultiplexing results by using sabre, yielding to a higher number of retained sequences and a higher number of SNPs in more individuals, and subsequently more statistical power. Second, rabbit populations across Australia show considerable population structure and several highly localized, strongly differential lineages (Iannella, Peacock, Cassey, & Schwensow, 2019). Using a double-digest GBS (Poland, Brown, Sorrells, & Jannink, 2012), Iannella et al. (2019) found a higher overlap in the genotyped SNPs across populations, suggesting that the use of two restriction enzymes is advantageous when comparing species with strong population differentiation across a larger geographical scale. Nevertheless, we found ten significant SNPs in common between both studies. Eight of the SNPs identified by the meta-analysis are in <40 kb distance of at least one gene known to have a role in virus infection, inflammation or immune reaction (Table 2) or are differentially expressed in acutely RHDV-infected rabbits (Schwensow et al., 2012).

Two candidate genes, ADGRL3 and GNB1 from the current data set as well as two further ones, OLFR64 and OR51I1 identified in our meta-analysis, share the GO term "G protein-coupled receptor signalling pathway (GO:0007186)" and are involved in signalling pathways that start from plasma, nuclear membrane or Golgi membranes (www.ensembl.org, downloaded 18.12.2019, Hunt et al., 2018). All known positive-strand RNA viruses replicate in association with remodelled host membranes of various organelles, for example Golgi, endoplasmic reticulum or endosomes (Mackenzie, 2005; summarized by Belov et al., 2007), and we also found significant enrichment of GO annotations connected with "Golgi" in our previous study (Schwensow, Detering, et al., 2017). Rearrangements of Golgi membranes indicating that RHDV uses host membranes have been reported (Urakova et al., 2015; Urakova, Strive, & Frese, 2017), and our results add evidence and suggest candidate genes for further investigating hypotheses that genes associated with pathways involving the Golgi apparatus are under RHD-driven selection. Interestingly, we found a strong decrease in linkage between SNPs in about 40 kb distance from the COP1 gene, and this pattern is suggestive of a combination of genotypes which have been selected against in the 16 years. Given that the predicted locations of the COP1 protein include the Golgi apparatus, this may be further evidence of RHDV-driven selective pressure.

For five of the presently identified candidate genes, information on their expression level is available. RPS6KA5, CEP78 and ESR1 were found to be downregulated and OR51I1 and RIC8B to be upregulated in rabbits acutely infected with RHD (Schwensow et al., 2012). RIC8B is guanine nucleotide exchange factor that plays a role in different cellular processes such as cell division, cell signalling, protein synthesis and embryo development in vertebrates (Maureira et al., 2016). OR51I1 is a member of the large gene family of olfactory receptors (Bulger et al., 2000), and notably, it was also significant in our meta-analysis, indicating its selective importance in both rabbit populations. The three downregulated genes have known roles in inflammatory processes or

virus infections. RPS6KA5 is a mitogen- and stress-activated protein kinase with functions in the regulation of inflammatory genes (Beck et al., 2008; Deak, Clifton, Lucocq, & Alessi, 1998), CEP78 is a protein required for the regulation of centrosome-related events during the cell cycle and was found to play a role in HIV pathogenesis (Hossain, Barbosa, Cohen, & Tsang, 2018), and ESR1 is a ligand-activated transcription factor that was found to be elevated in hepatitis B-related hepatocellular carcinoma (Dou, Fan, Cao, Yang, & Wang, 2016).

Given these multiple lines of evidence, we consider many of the identified genes as strong candidates for being under RHDV-driven selection. RHD occurs in our study area usually a few weeks after the breeding season has commenced (Mutze et al., 2002), and due to the relatively dry climate (annual rainfall of ca. 275 mm, predominantly in winter), the breeding season is mainly restricted to the wetter 4 months of the year. This means that our study period covered about 15–16 generations of rabbits. During this period, it can be assumed that RHDV has been the strongest viral selective force in the study population, although myxomatosis outbreaks occur in the Flinders Ranges as well. Clinical myxomatosis was only detected in <1% of individuals after RHDV was introduced (Mutze et al., 2002), but serology indicated that all older rabbits had been challenged with the disease as outbreaks occurred at least every second year between 1995 and 2002 (B. Cooke, unpublished data). In 1997, myxomatosis was estimated to have caused a mortality of about 50%, whereas RHDV killed approximately 90% of the susceptible antibody seronegative rabbits (B. Cooke unpublished data). Details on the mortality after that are not known, however, RHDV maintained low population numbers in the Flinders Ranges for nearly ten years (Mutze et al., 2014). An interaction between myxomatosis and RHD has been recognized for some time (Marchandeau et al., 2004). Pox viruses, including myxoma virus, suppress host immune responses (Kerr & McFadden, 2002), and this may continue even after rabbits have recovered from disease. Long-term studies have shown that earlier infection with myxoma virus reduces subsequent survival probability of rabbits in following RHD outbreaks (but not the reverse) (Wells et al., 2018). Other factors such as droughts, that also occurred between 1996 and 2012, may also have exerted selection. Interactions between different factors, as normal under selection regimes in nature, make it difficult to attribute selection pressure to one factor alone. However, RHDV has been the only new selective force post-1996 such that it is reasonable to assume that it has contributed strongly to the observed changes in the rabbit genomes that occurred in the 16 years covered by our study. Nevertheless, we cannot exclude that some of the results could also be explained by ongoing myxoma-rabbit co-evolution (Kerr et al., 2017) or may include genes that are broadly involved in generic resistance to pathogens.

Some information on candidate genes for myxoma virus (MYXV)-driven selection is available (Alves et al., 2019). They found a pattern of strong parallel evolution between rabbit populations in Australia, France and the United Kingdom and identified candidate genes for MYXV-driven selection. We found that one of the candidate genes identified in our meta-analysis (PAQR4) and one in the current study (ESRRG) was also in the list of genes suggested to be under MYXV-driven selection in the Australian population by Alves et al. (2019), albeit not among their most highly ranked genes. ESRRG encodes a ligand-independent orphan nuclear receptor that regulates a network of nuclear-encoded mitochondrial genes controlling oxidative metabolic function (Alaynick et al., 2007). PAQR4 is a member of the PAQR family, and the members within this family are involved in the regulation of several biological processes including metabolism and cancer and tumour development (Wu & Liu, 2019; Zhang et al., 2018). A role in virus infections in either of these two genes has, to our knowledge, not been described. Therefore, their potential roles in resistance against either of the viruses, or the most important selective agent, remain elusive.

Additional factors may, of course, have also influenced allele frequency changes over this period. Warren ripping was extensively conducted, and all known rabbit warrens on the Gum Creek station were destroyed by 2002 which would have had considerable impact not only on rabbit numbers but likely also on the population structure itself. Our estimates of the effective population size N_e indicate that the reproducing rabbit population on the Gum Creek station is now considerably smaller than it was in the pre-RHD era. N_e estimated from the 2012 samples was about half of the size it had been for the 1996 samples, and this may have caused the stronger genetic structure observed in 2012. While about half of the rabbits sampled in 2012 clustered closely with the same population as 1996 when using PCA, other individuals clearly separated from these along PC2 (Figure 2). This is intriguing because all individuals that were separated were sampled within the central zone of our study area (Figure 1). Rabbits sampled at the edges of the area clustered with the samples from 1996. One potential explanation for this may be that rabbits from adjacent areas have dispersed into the study area. However, RHDV would have had the same selective impact in all neighbouring populations as it is easily spread over vast distances, primarily through flying insects such as blowflies (Asgari, Hardy, Sinclair, & Cooke, 1998; Cooke, 2014; Schwensow et al., 2014). Even if the population was more structured in 2012, rabbits will have experienced RHDV-driven selection across the whole region. As we have excluded the SNPs that defined this structure in the 2012 population, this observation is unlikely to have affected our candidate SNP detection. Nevertheless, a change in the population structure may have had an additional selective impact in general through increasing population diversity.

We found evidence for selection on genes that were previously found to be differentially expressed in acutely infected versus healthy rabbits (Schwensow et al., 2012). There are, however, some differences between the temperate Turretfield population and the semi-arid Gum Creek population. First, our PCA showed clear genetic separation between both locations. Second, the Turretfield population consists of about 12 permanently inhabited warrens and some transitory holes in a 12-ha area somewhat isolated from other rabbit warrens by approximately 2 km of open sheep grazing paddocks (Peacock & Sinclair, 2009). In contrast, in the Gum Creek

area rabbits are widely spread over the landscape and likely form a larger, more continuous population (Jennings & Mutze, 2018). Our simulation study indicated that the number and genetic diversity of neighbouring populations on Gum Creek influence changes in gene frequency. A different metapopulation structure likely influences adaptive differentiation between populations (Fraser, Debes, Bernatchez, & Hutchings, 2014). Adaptive signatures of genes involved in pathogen resistance may differ markedly between populations under natural conditions and population-specific responses to co-evolving pathogens have been reported (Bankers et al., 2017). The virus interacts with its host at many different interfaces (e.g. during attachment, replication, maturation, release from host cells, interaction with the innate immune response), and that multiple mechanisms that could achieve the same resistance phenotype does seem not only feasible but likely.

Second, given the fast evolution of RHD in Australia (Eden, Read, Duckworth, Strive, & Holmes, 2015), different virus variants or outbreak frequencies may have exerted selection in both populations, which may have led to adaptations in different rabbit genes.

5 | CONCLUSION

Studying adaptation under natural conditions is important both to identify the relevant mechanisms and parameters in a variable environment and to test whether models developed in experimental approaches have the same importance in nature. Our approach allowed us to identify several genes potentially under RHDV-driven selection. Given the time span of 16 years that we covered in this study, the same study would not have been feasible in an experimental setting. Moreover, RHDV has evolved extremely quickly in Australia (Eden, Kovaliski, et al., 2015; Kovaliski et al., 2013), and only wild rabbits would reflect the corresponding co-evolutionary changes. This biological arms race between the rabbits and RHDV appears to keep overall RHD-induced mortality rates relatively stable over time (Wells et al., 2018). Choosing an approach that covered large parts of the rabbit genome with the availability of a sequenced and annotated rabbit reference genome allowed us to suggest interesting new candidate genes that may contribute to RHDV resistance. The meta-analyses revealed several SNPs with differential allele/genotype both in rabbits that died of RHD and those that survived, and which also differed in their frequency before RHDV was present in Australia and 16 years after selection by the virus. Experimental and functional validation will be an important next step in our efforts to understand the mechanisms behind RHDV infection and transmission. Comparing the allele frequencies of these candidate genes in other rabbit populations that have experienced different levels of RHDV impact would contribute to a better understanding of the relevance of these genes under natural conditions and may also help assessment of their potential to assist resistance to RHDV. Resequencing of genomes or exomes would help to learn more about the functionality of the detected candidate SNPs. It will be particularly important to investigate whether the here identified candidate genes also play a role in RHDV2 resistance, a new serotype virus that appeared in Australia in 2014 (Hall et al., 2015; Strive et al., 2020). RHDV2 has different biological properties compared to RHD such as the greater ability to infect young rabbits (Dalton et al., 2012; Neimanis, Pettersson, Huang, Gavier-Widén, & Strive, 2018), and it elucidates different transcriptional responses in the infected host (Neave et al., 2018). Understanding the co-evolutionary arms races between rabbits and RHDV (and RHDV2) may not only be important for Australian biocontrol programmes but also for counteracting the declining rabbit populations in southern Europe.

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AUTHOR CONTRIBUTIONS

NS, BC and PC designed the study. PC supervised the project. SP performed the bioinformatic and statistical analyses. NS performed the lab work, analysed the data and wrote the manuscript. DP and BC provided the samples. All authors critically revised and approved the manuscript.

DATA AVAILABILITY STATEMENT

All scripts publically available at https://uofabioinformaticshub.github.io/2014_SchwensowGBS/. Sequencing data are available at Dryad (https://doi.org/10.5061/dryad.2ngf1vhjr).

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SUPPORTING INFORMATION

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

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