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1 **Frequent intergenotypic recombination between the non-structural and structural**
2 **genes is a major driver of epidemiological fitness in caliciviruses**

3

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19 **Abstract**

20 The diversity of lagoviruses (*Caliciviridae*) in Australia has increased considerably. By
21 the end of 2017, five variants from three viral genotypes were present in populations of
22 Australian rabbits, while prior to 2014 only two variants were known. To understand the
23 interactions between these lagovirus variants we monitored their geographical
24 distribution and relative incidence over time through a landscape-scale competition
25 study, and from this, revealed potential drivers of epidemiological fitness. Within three
26 years of the arrival of GI.1bP-GI.2 (RHDV2) into Australia, we observed the emergence
27 of two novel recombinant lagovirus variants, GI.4eP-GI.2 (4e-recombinant) in New
28 South Wales and GI.4cP-GI.2 (4c-recombinant) in Victoria. Although both novel
29 recombinants contain the non-structural genes from benign, rabbit-specific, enterotropic
30 viruses, these variants were recovered from the livers of both rabbits and hares that had
31 died acutely. This suggests that determinants of host and tissue tropism for lagoviruses
32 are associated with the structural genes, and that tropism is intricately connected with
33 pathogenicity. Phylogenetic analyses demonstrated that the 4c-recombinant emerged
34 independently on multiple occasions, with five distinct lineages observed. Both new
35 recombinant variants replaced the previous dominant parental RHDV2 in their
36 respective geographical areas, despite sharing an identical or near-identical (i.e., single
37 amino acid change) major capsid protein with the parental virus. This suggests that
38 epidemiological fitness of these recombinants was not driven by antigenic variation in
39 the capsid, implicating the non-structural genes as key drivers of epidemiological
40 fitness. Molecular clock estimates place the GI4.e recombination event in early to mid-
41 2015, while the five GI.4c recombination events occurred from late 2015 through to
42 early 2017. The emergence of at least six viable recombinant variants within a two-year
43 period highlights an unprecedented frequency of these events, detectable only due to
44 intensive surveillance, and demonstrates the importance of recombination in lagovirus
45 evolution.

46

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47 **Introduction**

48 Caliciviruses are an important group of vertebrate-infecting viruses. They include
49 noroviruses, the major cause of gastroenteritis in humans worldwide [1], and members
50 of the genus *Lagovirus*, which infect rabbits and hares. Some lagoviruses are
51 hepatotropic and cause an acute fulminant viral hepatitis with a case fatality rate
52 exceeding 90%, while others are enterotropic and are thought to be entirely benign [2].
53 These are referred to as rabbit haemorrhagic disease viruses (RHDVs) and rabbit
54 caliciviruses (RCVs), respectively.

55 Lagoviruses are hierarchically classified by their major capsid protein (VP60) type, and
56 less frequently by polymerase type, into genogroups (e.g. GI, GII), genotypes (e.g. GI.1,
57 GI.2, GI.4), and variants (e.g. GI.1a, GI.1b, GI.1c) [3]. The first lagoviruses identified,
58 from hares in Europe in the early 1980s, were those of the GII.1 genotype (EBHSV) [4].
59 Subsequently, mortality events in *Oryctolagus* rabbits in China in 1984 led to the
60 discovery of genotype GI.1 (RHDV1) viruses [5]. With an increasing diversity of GI.1
61 viruses, this genotype was further subdivided based on the VP60 phylogeny into several
62 variants. In 1997, the first antigenic variants of GI.1 viruses, now classified as GI.1a
63 (RHDVa), were described in Italy [6] and these spread throughout Europe and more
64 distantly. Then, in 2010, the GI.2 (RHDV2) variant first emerged in France [7-9]. RHDV2
65 rapidly spread globally, triggering epizootics worldwide in wild and domestic lagomorph
66 populations. As surveillance efforts and technologies improved, non-pathogenic GI.3
67 (RCV-E1) and GI.4 (RCV-A1, RCV-E2) lagoviruses were identified in the 1990s, first in
68 Europe [10-13] and then in Australia [14] and New Zealand [15]. Non-pathogenic hare
69 lagoviruses (GII.2, GII.3, GII.4, GII.5; HaCV) have also been reported more recently, in
70 2014 in Europe [16-18] and 2019 in Australia [19].

71 Lagoviruses, like other caliciviruses, are small, non-enveloped viruses containing a
72 monopartite positive-sense single-stranded RNA genome approximately 7.5 kb in length
73 (Figure 1) [2]. Virus particles contain both genomic RNA (gRNA) and 3' co-terminal sub-
74 genomic RNAs (sgRNA) approximately 2.5 kb in length [20]. In lagoviruses, the non-
75 structural (NS) genes are situated upstream of the major capsid protein, VP60, all of
76 which are encoded as a single large polyprotein (ORF1), while the 3' terminal ORF2

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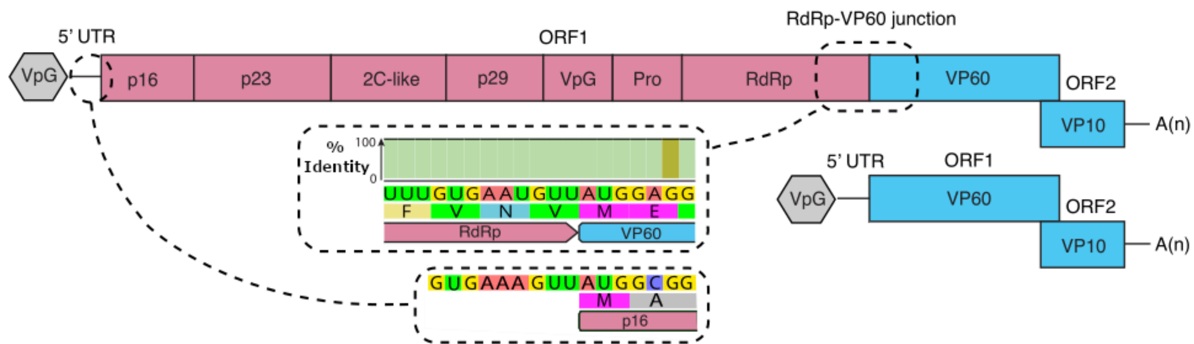
77 encodes a minor structural protein that is presumed to be important for genome release
78 during infection [21]. Lagoviruses encode seven NS proteins, including a helicase (2C-
79 like), a viral genome-linked protein (VpG), a protease (Pro), an RNA-dependent RNA
80 polymerase (RdRp), and three proteins of unknown function (p16, p23, p29). Both
81 gRNA and sgRNA are linked to VpG at their 5' end and are polyadenylated at their 3'
82 end [22]. There is considerable homology between the 5' terminal nucleotide sequences
83 of the gRNA and sgRNA (Figure 1) [22], facilitating recombination at the junction
84 between the RdRp and VP60; this region is highly conserved within and between
85 genogroups.

86



87

88 **Figure 1: Lagovirus genome organisation.** Genomic RNAs (gRNA) encode the non-
89 structural proteins (pink) and structural proteins (blue), while subgenomic RNAs
90 (sgRNA) encode the structural proteins only. Both RNAs are linked to VpG at their 5'
91 end and are polyadenylated at their 3' end. Inset boxes demonstrate the nucleotide
92 sequence identity between the 5' untranslated region (UTR) of the gRNA and the RdRp-
93 VP60 junction. An identity plot (based on n = 478 near-complete GI sequences from
94 GenBank; green indicates 100% identity) is also shown for the junction region.

95

96 Recombination is an important evolutionary mechanism in many RNA viruses [23]. In
97 the *Caliciviridae*, recombination frequently occurs at the junction between the NS and
98 structural genes, effectively mixing a set of structural genes with an entirely new set of
99 NS genes [24-26]. Recombinant lagoviruses are defined by the nomenclature [RdRp

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100 genotype]P-[capsid genotype], and can include combinations of two pathogenic RHDVs
101 (e.g. GI.1bP-GI.2) as well as the seemingly more common combination of a benign
102 RCV and a pathogenic RHDV (e.g. GI.4eP-GI.1a, GI.4eP-GI.2, GI.3P-GI.2) [24, 27-32].
103 Retrospective phylogenetic analyses have demonstrated that all GI.2 viruses so far
104 described are recombinants (GI.3P-GI.2), implying that GI.2 is an orphan capsid-type
105 [24]. Intergenogroup recombinants between GI (rabbit and hare) and GII (hare) viruses
106 have also been reported, with two GII.1P-GI.2 viruses recovered from hares in Germany
107 in 2014 and 2019 [33].

108 Prior to 2016, three lagovirus genotypes (GI.1, GI.2, GI.4) from the G.I genogroup,
109 comprising five distinct variants — GI.4 (RCV-A1), GI.1c (RHDV1), GI.1a (RHDVa-K5),
110 GI.4eP-GI.1a (RHDVa-Aus), GI.1bP-GI.2 (RHDV2) — had been reported in Australia.
111 These variants differ in their host and tissue tropism and in pathogenicity [34]. RCV-A1
112 is a benign enterotropic virus that has circulated in wild and domestic rabbits, likely
113 since at least the 1950s [14, 35]. RHDV1 and RHDVa-K5 are pathogenic viruses, both
114 deliberately released [36]. RHDVa-Aus and RHDV2 were both exotic incursions, first
115 detected in January 2014 and May 2015, respectively [32, 37]. Phylogenetic analyses
116 suggest that both incursions arose from single point source introductions with
117 subsequent ongoing transmission in Australian rabbit populations [32, 34]. In mid-2017
118 a novel GI.4eP-GI.2 recombinant virus (4e-recombinant) was detected, comprising the
119 NS sequences of the RHDVa-Aus virus and RHDV2 VP60 sequences [31]. However,
120 the long-term epidemiological significance of this recombinant variant was unclear.

121 Australia is an ideal setting for understanding the evolution, recombination, and
122 epidemiological interactions of caliciviruses. Australia is an isolated landmass with a
123 large rabbit population that is distributed across most of the country. The genetic
124 diversity of Australian lagoviruses arose from a small number of introduction (or
125 incursion) events, and the parental sequence as well as the timing of the introduction is
126 known in several instances. Since there is little movement of rabbits into the country,
127 the study of lagoviruses can occur in a relatively controlled environment with a defined
128 number of pre-existing pathogenic viruses. Additionally, a robust surveillance system for
129 these viruses has been in place since 2014. The previous lack of diversity of the

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130 Australian lagovirus population and sudden diversification of lagovirus variants in
131 2014/2015 created the perfect natural experimental conditions for monitoring the
132 frequency of successful recombination and drivers of viral evolution. In this study we
133 monitored the epidemiological and evolutionary dynamics of existing lagovirus variants
134 in Australia, while determining the frequency and importance of successful
135 recombination events between these viruses.

136

137 **Materials and Methods**

138 *Sample submission*

139 Samples from dead domestic and wild lagomorphs (i.e., rabbits and hares) were
140 submitted either directly or via RabbitScan (https://www.feralscan.org.au/rabbitscan/) to
141 the Commonwealth Scientific and Industrial Research Organisation (CSIRO) by
142 veterinarians, pet owners, landholders, and members of the public as part of ongoing
143 opportunistic lagovirus surveillance. No animal ethics approvals are required for
144 sampling rabbits that are found dead in Australia. Samples were provided either fresh-
145 frozen or stored in an RNA stabilization solution [31]. Additionally, 42 positive samples
146 submitted to the Elizabeth Macarthur Agricultural Institute (EMAI) for RHDV diagnostic
147 testing were forwarded to CSIRO, either as fresh-frozen tissue or as a swab of the cut
148 tissue stored in phosphate buffered gelatin saline (PBGS) [38]. Samples for this study
149 were restricted to those collected between 1 January 2014 (based on the estimated
150 time of incursion of GI.1bP-GI.2 into Australia [34, 39]) and 30 September 2020, and
151 were received from the Australian Capital Territory (ACT, n = 139), New South Wales
152 (NSW, n = 401), the Northern Territory (NT, n = 7), Queensland (QLD, n = 86), South
153 Australia (SA, n = 227), Tasmania (TAS, n = 221), Victoria (VIC, n = 649), and Western
154 Australia (WA, n = 194) (total n = 1,924).

155 *Initial testing*

156 RNA was extracted from 20 – 30 mg of tissue, predominantly liver or bone marrow, but
157 occasionally spleen, kidney, muscle, ear tip, eyelid, or blowfly maggots retrieved from
158 the carcass. After homogenisation with glass beads using a Precellys 24-dual tissue

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159 homogenizer (Bertin Technologies), RNA was extracted using either the Maxwell 16
160 LEV simplyRNA tissue kit (Promega, Alexandria, NSW) or RNeasy mini kit (Qiagen,
161 Chadstone Centre, VIC) as per manufacturers' directions. For swab samples, RNA was
162 extracted from 200 µl PBGS using the Purelink viral RNA/DNA mini kit (ThermoFisher
163 Scientific). Samples were screened for known lagoviruses using a broadly-reactive
164 lagovirus SYBR-green based RT-qPCR targeting VP60 and sgRNA, as described
165 previously [31].

166 *Variant identification*

167 Of the samples that were positive by screening RT-qPCR (n = 1,209), 175 were
168 sequenced previously for other studies [31, 32, 34, 37, 40]. The remaining samples
169 were screened for recombination by RT-PCR and sequencing. RT-PCR primers were
170 designed that spanned the RdRp-VP60 junction, generating a 555 bp amplicon (Rec2
171 RT-PCR; Supplementary table 1). Primers were subsequently modified by the 5'
172 addition of Illumina pre-adapter and index primer binding sequences to enable high-
173 throughput Illumina sequencing (Rec2.tailed RT-PCR; Supplementary table 1). Assays
174 were experimentally validated to confirm reactivity with all known Australian lagoviruses.
175 For a subset of samples (prior to the development of the Rec2 RT-PCR), amplification
176 over the RdRp-VP60 junction was performed with alternative primer sets
177 (Supplementary table 1). Briefly, each 25 µl PCR reaction comprised 1x OneStep
178 Ahead RT-PCR master mix (Qiagen), 1x OneStep Ahead RT mix (Qiagen), 0.5 µM each
179 primer, and 1 µl of extracted RNA diluted 1/10 in nuclease-free water. Cycling
180 conditions were: 45 °C for 15 min, 95 °C for 5 min, followed by 40 cycles of 95 °C for 15
181 sec, 55 °C for 15s, 68 °C for 2 min, with a final extension of 68 °C for 5 min.

182 Amplicons were purified and either sent for Sanger sequencing at the Biomolecular
183 Resource Facility, The John Curtin School of Medical Research, Australian National
184 University, or were indexed, pooled, and sequenced on an Illumina MiSeq (300-cycle v2
185 chemistry) according to the manufacturer's directions. After quality trimming, sequences
186 were partitioned into regions upstream and downstream of the RdRp-VP60 junction.
187 Each partition was aligned with representative sequences of known Australian lagovirus
188 variants using MAFFTv7.450 [41] in Geneious Prime 2020.0.4, and an RdRp and VP60

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189 variant type was assigned for each sample. Four isolates were identified as mixed
190 infections based on sequencing. Alignments of partial sequences are available at
191 https://doi.org/10.25919/758f-4t15.

192 *Molecular epidemiological analyses*

193 To investigate the geographical distribution and relative incidences of recombinant
194 variants over time, positive samples were restricted to those reported after 1 January
195 2016; i.e., 6 months prior to the first detection of a novel recombinant (n = 1,139).
196 Samples identified as RHDVa-K5 (n = 121) were excluded from further analyses since
197 these were mostly associated with release sites of this approved biocontrol virus; that is,
198 this variant was not transmitting through rabbit populations and was therefore not
199 competing epidemiologically with other virus variants [39]. Samples from the ACT were
200 grouped with those from NSW, since the ACT is a small jurisdiction (2,358 km^2) located
201 within NSW and was considered epidemiologically to be part of NSW.

202 For the 4c-recombinant lineage designation, 169 4c-recombinant sequences spanning
203 the RdRp-VP60 junction were aligned with 77 4c-recombinant full genomes and 26
204 publicly available GI.4 genomes using MAFFTv7.450 [41]. The taxa included in the final
205 data set are detailed in Supplementary table 2. The alignment was trimmed to
206 nucleotide positions 5,115 – 5,619, based on the M67473.1 DEU/FRG/1988.50
207 reference sequence (https://www.ncbi.nlm.nih.gov/genbank/). The remaining 109 4c-
208 recombinant sequences obtained in this study were not suitable for inclusion in the final
209 alignment because they were generated either using Illumina sequencing or an
210 alternative primer set and thus did not span the complete region of the alignment. Model
211 selection and maximum likelihood (ML) tree inference was conducted using IQ-
212 TREEv1.6.11 [42], and branch supported was estimated by 1,000 replicates of the SH-
213 like approximate likelihood ratio test [43]. The tree was rooted on the internal branch
214 leading to the RCV-A1 lineage.

215 *Full genome sequencing*

216 Of the 1,034 samples genotyped to the variant level, a subset of 224 were selected for
217 full genome sequencing (Supplementary table 3). These samples were selected to be

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218 geographically and temporally representative, with a focus on the novel 4e- and 4c-
219 recombinants. Viral genomes were amplified in overlapping fragments, and DNA
220 libraries were prepared and sequenced using Illumina Miseq technology as described
221 previously [35, 40]. Primers used for amplification of the overlapping fragments are
222 detailed in Supplementary table 1. Consensus sequences were constructed by mapping
223 cleaned reads to the lagovirus GI reference sequence (GenBank accession M67473.1
224 DEU/FRG/1988.50) using Geneious Prime 2020.0.4. Sequences were deposited in
225 GenBank under accession numbers MW460205 – MW460242.

226 *Recombination analysis*

227 To further characterise the putative novel 4c-recombinant variant, and the newly
228 sequenced GI.4e-recombinants, recombination analyses were conducted on full
229 genome sequences using the RDP4 software [44]. The complete genome alignment
230 included lagovirus GI potential parent sequences from GenBank
231 (https://www.ncbi.nlm.nih.gov/genbank) (n = 384, 7,309 nt, Supplementary table 4).
232 Recombination was reported if detected by at least two of three primary scanning
233 methods (RDP, MaxChi and GENECONV), with a highest acceptable p-value of 0.05
234 with Bonferroni multiple comparison correction. The BootScan, 3Seq, CHIMAERA, and
235 SciScan methods were used to verify recombination events identified using the primary
236 methods.

237 *Phylogenetic analyses*

238 Newly sequenced lagovirus genomes were aligned with representative GI lagovirus
239 sequences from GenBank (https://www.ncbi.nlm.nih.gov/genbank) using MAFFTv7.271
240 [41]. The complete genome alignment was split into two data sets representing (i) the
241 NS genes (n = 240 sequences, 5,231 nt, Supplementary table 5), and (ii) the VP60
242 structural gene (n = 272, 1,740 nt, Supplementary table 6). An additional VP60
243 alignment was constructed that contained the VP60 of the newly sequenced viruses
244 along with all published Australian RHDV2 VP60 sequences (n = 332, 1,737 nt,
245 Supplementary table 7). Model selection and ML tree estimation was performed using
246 IQ-TREEv1.6.11 [42], as described above. Ancestral state reconstruction to identify
247 nonsynonymous changes along individual branches was carried out using PAMLv4.9 [45].

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248 *Phylogeographic analyses*

249 A Bayesian Markov chain Monte Carlo (MCMC) approach was employed to infer time-
250 scaled phylogenies, and from this, to infer the temporal pattern and most likely
251 geographic location of internal nodes (i.e., phylogeography). All data sets were initially
252 screened using TempEst [46] to ensure that sufficient temporal signal was present,
253 using ML phylogenies as input to construct linear regressions of root-to-tip genetic
254 distances against sampling time. Due to recombination between the NS and capsid
255 genes, two data sets either side of the recombination breakpoint were analysed
256 separately: (i) a VP60 gene data set containing all published Australian GI.2 capsid
257 sequences together with the capsid genes of the recombinants sequenced here (n =
258 332, 1,737 nt, Supplementary table 7); and (ii) an NS genes data set of all published
259 Australian GI.4 sequences together with the NS genes of the recombinants sequenced
260 here (n = 188, 5,218 nt, Supplementary table 8). The Bayesian Evolutionary Analysis by
261 Sampling Trees (BEAST) software v1.8 [47] was used to conduct Bayesian MCMC
262 analysis of each data set, using substitution models inferred using ModelFinder as
263 implemented in IQ-TREEv1.6.11 [42] (VP60, SYM+G4; NS, SYM+I+G4) [48]. A discrete
264 traits partition indicating the sampling location (Australian state) was included to
265 facilitate ancestral state reconstruction of location (utilizing the symmetric substitution
266 model, inferring social network with Bayesian Stochastic Search Variable Selection, and
267 a strict clock model). All analyses were run twice to convergence (defined as an
268 effective sample size >200) to confirm consistency. Marginal likelihood estimation using
269 path sampling/stepping-stone sampling was used to assess the most appropriate clock
270 model prior (strict vs uncorrelated log-normally distributed [UCLD]) and tree prior
271 (Gaussian Markov random field Bayesian skyride model vs constant size coalescent vs
272 exponential coalescent) for the nucleotide partition.

273 The NS data set had a better temporal signal (R-squared of 0.86 on linear regression of
274 root-to-tip genetic distance against sampling time) and a larger sampling window
275 compared to the VP60 data set and was therefore considered the most informative. The
276 UCLD clock model and constant population size coalescent tree prior had the highest
277 marginal likelihood for this data set.

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278 *Figures*

279 Figures were constructed using R v4.0.3 [49] using the following packages: 'ggtree
280 v2.2.1' [50], 'treeio v1.12.0' [51], 'scales v1.1.1' [52], 'tidyverse v1.3.0' [53], 'ape v5.4'
281 [54], 'phytools v0.7-47' [55], 'phangorn v2.5.5' [56], 'cowplot v1.0.0.9000' [57], 'lubridate
282 v1.7.8' [58], 'plyr v1.8.6' [59], and 'ozmaps v0.3.6' [60]. Figure 3B was generated in
283 GeneiousPrime 2020.2.4 using 3-D structure viewer.

284

285 **Results**

286 *Phylogenetic analyses reveal five independent recombination events in GI.4cP-GI.2*
287 *viruses*

288 A recombinant lagovirus variant (GI.4eP-GI.2; 4e-recombinant) was previously detected
289 in Australia using a multiplex RT-PCR assay. This assay is only able to detect specific
290 recombinant variants [31]. Therefore, to comprehensively screen for lagovirus
291 recombination events we sequenced the RdRp-VP60 junction region (i.e., the typical
292 recombination breakpoint) of 1,034 lagoviruses collected between January 2014 and
293 September 2020 (inclusive) from wild and domestic lagomorphs found dead across
294 Australia. This screen identified six distinct lagovirus variants circulating in Australia
295 during the study period: four known variants (GI.1cP-GI.1c (RHDV1), GI.1bP-GI.2
296 (RHDV2), GI.1aP-GI.1a (RHDVa-K5), GI.4eP-GI.1a (RHDVa-Aus)), the previously
297 reported 4e-recombinant (GI.4eP-GI.2), and a novel GI.4cP-GI.2 putative recombinant
298 (4c-recombinant).

299 We selected a subset of novel 4c-recombinant viruses, recently emerged 4e-
300 recombinant viruses, and parental RHDV2 viruses for full genome sequencing, selecting
301 isolates that were representative of the temporal and geographical spread of these
302 variants. Recombination analyses of newly sequenced 4e-recombinant viruses
303 confirmed these as recombinants with a breakpoint at the RdRp-VP60 junction,
304 supporting our previous finding (Table 1, Supplementary table 4). Recombination
305 analyses of the novel putative 4c-recombinant viruses also detected a recombination
306 breakpoint at the typical RdRp-VP60 junction (Table 1). The most likely parental

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307 variants for the 4c-recombinant, as determined by the RDP4 software, were benign
308 Australian GI.4 (RCV-A1) lagoviruses in the NS region (GenBank accession KX357699)
309 and Australian GI.2 (RHDV2) viruses in the VP60 region (GenBank accession
310 MF421577), strongly suggesting that this recombination event occurred within Australia
311 after the arrival of GI.2 in late 2014.

312 Subsequently, the nucleotide alignment was partitioned either side of the putative
313 breakpoint and ML phylogenetic trees were inferred for the NS genes and the VP60
314 gene separately. There was clear incongruence between the NS and VP60
315 phylogenies, supporting the results of the RDP4 analysis (Figure 2). Strikingly, ML
316 phylogenies based on the VP60 region revealed five distinct lineages of the 4c-
317 recombinant variant, suggesting multiple independent emergences of this variant
318 (Figure 3A). In contrast, the NS gene sequences from this variant formed a
319 monophyletic group (collapsed in Figure 2A). All 4e-recombinant sequences fell within a
320 single clade in both trees (Figure 2).

321

322

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323 **Table 1. Recombination analyses**

Recombinant details		Putative parent^		P-value for recombination event by detection method		
Representative Recombinant Sequence	**Breakpoint Position***	**NS Parent**	**S parent**	**RDP**	**Maxchi**	**GENE-CONV**
GI.4eP-GI.2/O.cun*/AUS/ NSW/2017-03-17/CAR-8	5306	KY628315/ GI.4eP-GI.1a	MF421585/ GI.1bP-GI.2	4E-194	3E-43	3E-189
GI.4cP-GI.2/O.cun/AUS/ VIC/2017-02-05/ALV-1	5307	KX357699/ GI.4c	MF421577/ GI.1bP-GI.2	3E-148	2E-39	1E-154

324 A more detailed version of this table can is supplied as Supplementary table 4.

325 *Nucleotide position of putative breakpoint according to M67473.1/DEU/FRG/1988.50 reference sequence
326 numbering.

327 ^NS refers to putative parent providing the non-structural genes; S refers to putative parent providing the structural
328 genes.

329

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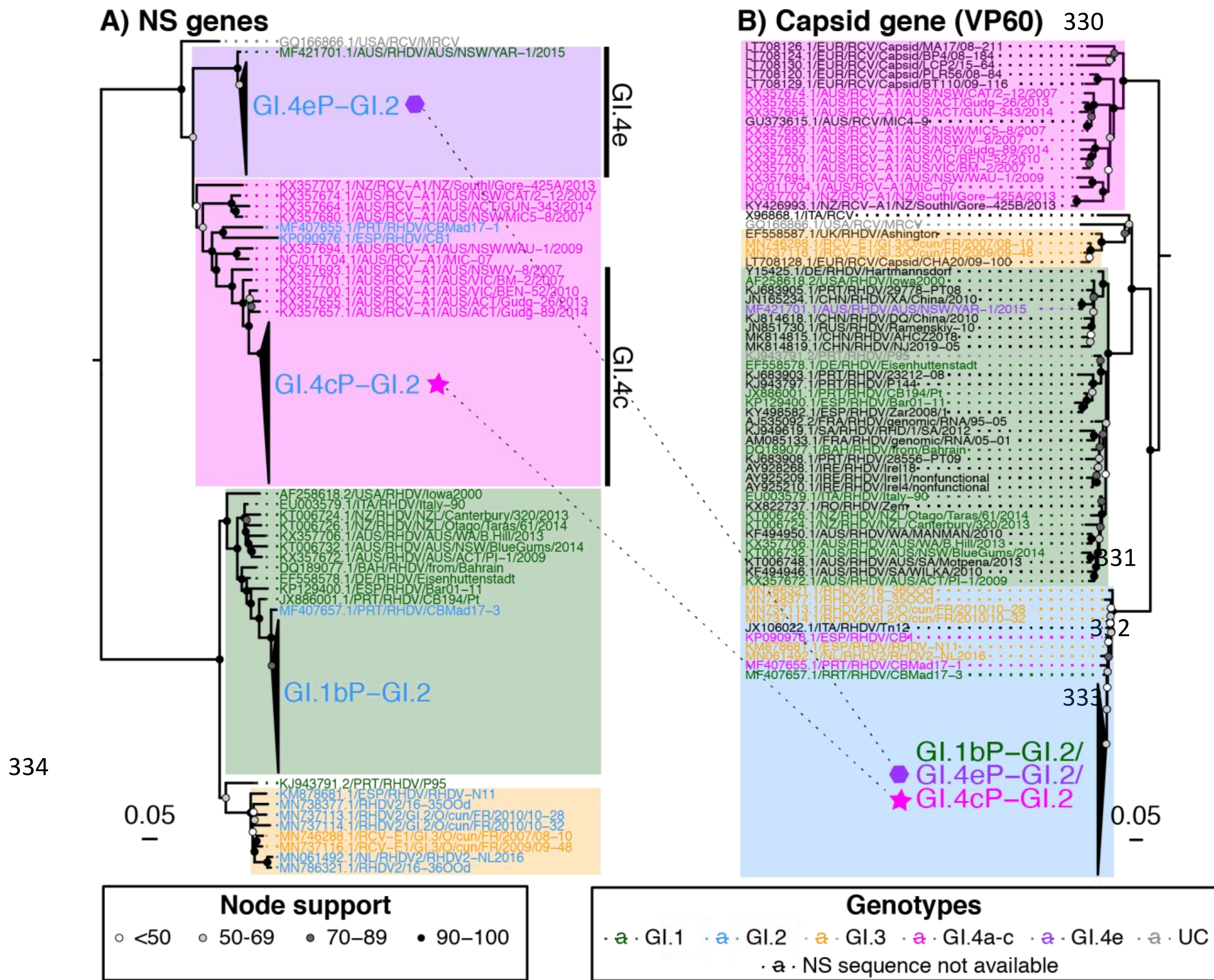

331

332

333

334

335 **Figure 2. ML phylogenetic trees of the (A) NS genes and (B) the VP60 gene of**
336 **representative lagovirus sequences and the Australian recombinant sequences.**
337 Discrepancies between the highlight and text colour indicate recombinant sequences.
338 Clades are highlighted according to the genotype of the genetic segment analysed in
339 the tree, while taxa names are coloured by the genotype of the alternative segment (i.e.,
340 taxa names in the NS gene tree are coloured by the genotype of the capsid gene, and
341 vice versa). Taxa names coloured black indicate that the NS sequence data is
342 unavailable. UC, unclassified. Major clades have been collapsed for visualization
343 purposes. All Australian recombinant sequences from this study fall within the collapsed
344 clades indicated by the star (GI.4cP-GI.2) or hexagon (GI.4eP-GI.2) symbols. The
345 dotted lines between the two trees link the Australian recombinant clades. Phylogenies

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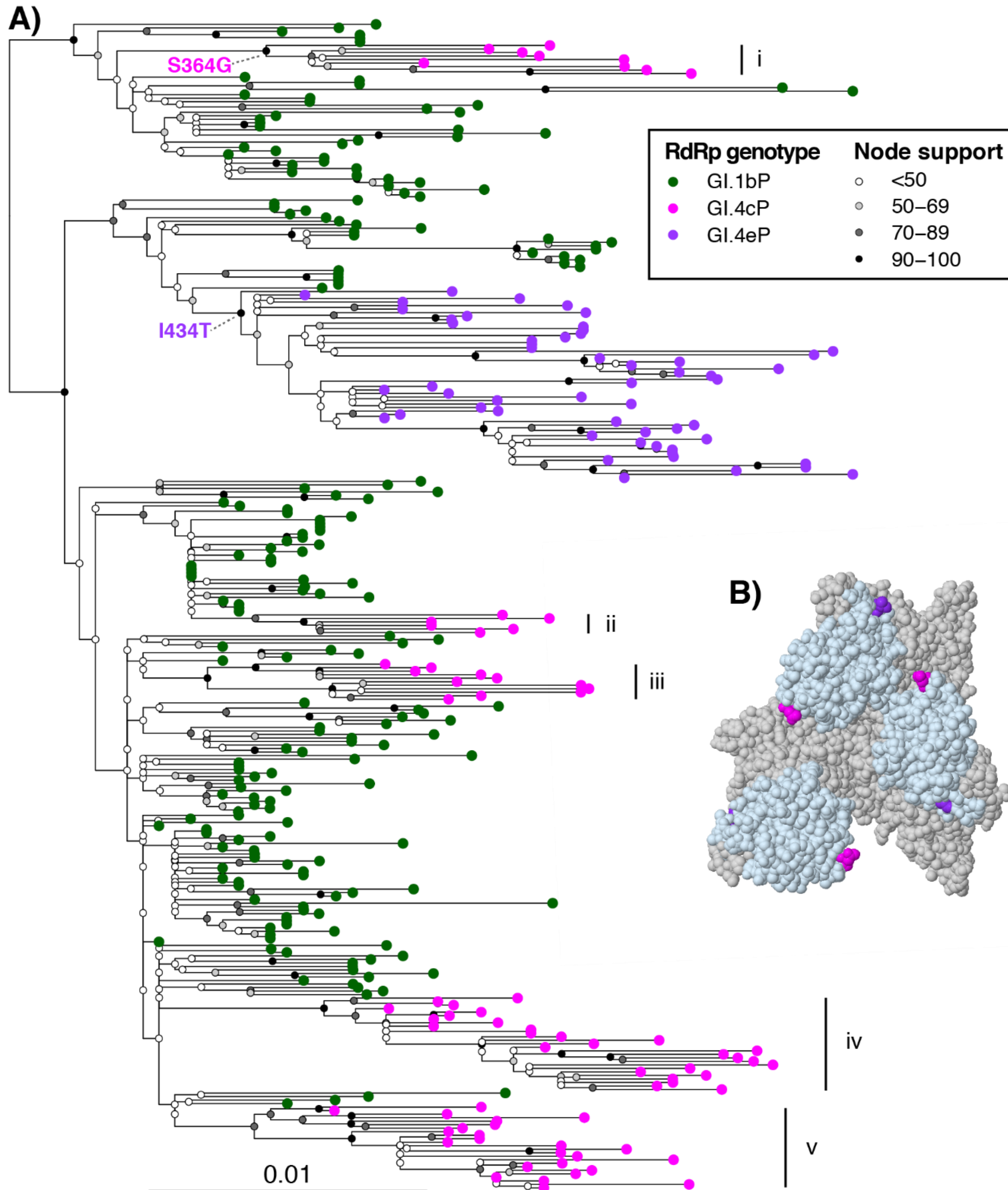
346 were midpoint rooted and the scale bars represent the number of substitutions per site.
347 Bootstrap support values (1,000 replicates) are indicated by shaded circles at the
348 nodes.

349



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351 **Figure 3**: **ML phylogenetic tree of Australian GI.2 VP60 sequences and amino acid**
352 **changes associated with the emergence of 4c- and 4e-recombinant variants.** (A) A
353 phylogeny was inferred from the VP60 gene sequences (n = 332) of newly sequenced
354 Australian GI.2 lagoviruses (including recombinants) along with representative
355 published sequences. Coloured tip points represent the polymerase (RdRp) genotype:
356 GI.1bP, green; GI.4cP, pink; GI.4eP, purple. GI.4cP lineages are labelled (i through v).
357 The phylogeny was midpoint rooted and the scale bar represents nucleotide
358 substitutions per site. Non-parametric bootstrap support values (1,000 replicates) are
359 indicated by shaded circles at the nodes. Associated metadata including taxon names
360 are provided in Supplementary table 7. Ancestral state reconstruction to identify
361 nonsynonymous changes associated with internal divergence events was conducted
362 using PAML. All changes occurring on branches leading to the GI.4cP and GI.4eP
363 lineages are labelled at the relevant node. (B) These changes were mapped to an
364 asymmetric unit of the atomic model of lagovirus GI VP60 (PDB accession: 3J1P). The
365 P2 domain is coloured in light blue. Amino acid 364, associated with the change in 4c-
366 recombinant lineage i, is coloured pink and 434, associated with the 4e-recombinant, is
367 coloured purple.

368

369 *Rapid epidemiological replacement of parental RHDV2 by the newly emerged 4c- and*
370 *4e-recombinants*

371 Detections of the new 4c-recombinant variant consistently outnumbered those of the
372 parental RHDV2 in VIC by April 2018, 14 months after the first detection in Alvie, VIC,
373 on 5 February 2017, suggesting that this variant was outcompeting previously
374 circulating viruses (Figure 4). This pattern of emergence and replacement was also
375 observed in TAS (Figure 4). This variant was detected sporadically in NSW/ACT from
376 October 2017 where it cocirculated with the locally dominant 4e-recombinant; notably,
377 the proportional incidence of the 4c-recombinant variant has increased in this region
378 during 2020. Interestingly, a single detection of the 4c-recombinant was identified in WA
379 in November 2018 in two domestic rabbits recently imported from TAS as breeding
380 stock. The sequence of this 4c-recombinant virus detected in WA clearly nested within

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381 the genetic diversity found in TAS (see Figure 4A, clade v), suggesting a single
382 incursion into WA with no ongoing local transmission. During the study period, the novel
383 4c-recombinant was recovered from several juvenile animals less than 800 g
384 bodyweight and as young as 4 weeks old, demonstrating pathogenicity in young rabbits,
385 like all other GI.2 variants characterized so far. It was also identified in one European
386 brown hare (*Lepus europaeus*).

387 We also examined the distribution of the previously reported 4e-recombinant variant
388 [31]. Most detections (n = 140) were identified in NSW/ACT. The earliest detection was
389 in Tubbul, NSW, on 13 July 2016, and by March 2017 it had mostly replaced the
390 parental RHDV2 to become the dominant lagovirus variant in this region (Figure 4B).
391 More recently, in late 2019 and throughout 2020, it has cocirculated with the 4c-
392 recombinant in NSW/ACT. Although most detections were in adult rabbits, the 4e-
393 recombinant was also recovered from three European brown hares and from several
394 juvenile rabbits down to 250 g bodyweight, suggesting a similar host tropism to the
395 parental RHDV2 virus and the novel 4c-recombinant.

396

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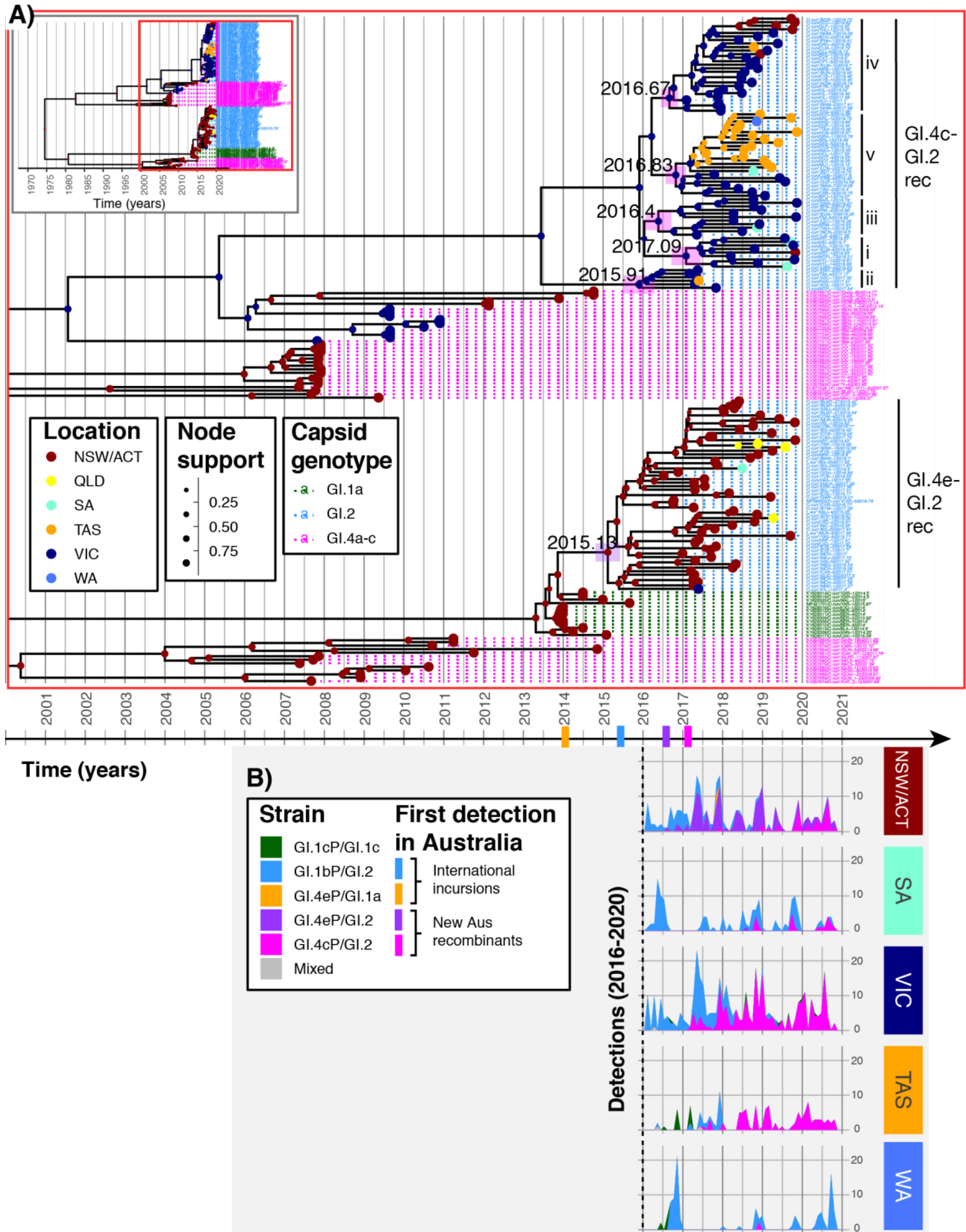

397 **Figure 4: Timing and location of the emergence of Australian lagovirus**
398 **recombinants based on time-structured phylogeographic reconstruction and RT-**

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399 **qPCR detections.** Panels A and B are scaled to the same x-axis (shown between the
400 two panels), given as time in years. (A) Time-structured phylogeographic analysis of
401 Australian GI.4 lagovirus NS genes (uncorrelated log-normally distributed clock model,
402 constant tree prior). The inset indicates the section of the phylogeny shown. Node and
403 tip points are coloured according to location (Australian state) and node points are sized
404 according to posterior support for that clade. Taxon names are coloured by capsid
405 variant. The mean time to most recent common ancestor for the GI.4eP-GI.2 and each
406 GI.4cP-GI.2 recombinant lineage is indicated at the respective internal node, and
407 horizontal bars at these nodes represent the 95% highest posterior density (HPD). The
408 recombinant (rec) clades are labelled, including the GI.4cP-GI.2 lineages (i – v). (B)
409 Lagovirus positive samples collected in NSW/ACT, SA, VIC, TAS, and WA from 2016 to
410 2020 (n = 739) were genotyped to the variant level by sequencing either side of the
411 typical calicivirus recombination breakpoint. The number of detections of each variant
412 by month are shown for each geographical region as an area plot, with the plotted area
413 coloured by variant.

414

415 *All recombination events occurred within a two-year timeframe in eastern Australia*

416 To determine the temporal dynamics, the location of emergence, and the rate of viable
417 recombination events in Australian lagoviruses, we conducted time-structured
418 phylogenetic analyses for the NS and capsid genes of recombinant viruses and related
419 sequences (i.e., Australian GI.2 VP60 and GI.4 NS sequences). Based on regression
420 analysis, data from the GI.4 NS dataset was considered more accurate and is therefore
421 presented in Figure 4. Based on these analyses, the 4e-recombinant was the first
422 variant to emerge, with the most recent common ancestor of this lineage dated to early
423 to mid-2015 (95% highest posterior density [HPD] NS dataset, 2014.8 – 2015.43)
424 (Figure 4A). For the 4c-recombinants, the 95% HPD intervals of the time to most recent
425 common ancestor (TMRCA) overlapped between lineages, making it difficult to
426 confidently define the exact timings and order of emergence of each lineage (Figure
427 4A). However, the data clearly show that these five recombination events all occurred
428 within the space of two years. This two-year timeframe was consistently observed

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429 regardless of whether the analysis was conducted on the NS genes GI.4 data set or the
430 VP60 gene GI.2 data set. Our phylogeographic analysis (Figure 4A) suggests that all
431 the 4c-recombinant lineages emerged in VIC (probability >0.99 for all lineages), with
432 subsequent spread to other states. Notably, this was observed even for lineage v, which
433 was first detected in Tasmania. In contrast, the 4e-recombinant clearly emerged in
434 NSW/ACT (probability >0.99, Figure 4A); this is further supported by our
435 epidemiological data, where we found only limited detections of this variant in other
436 states (QLD, VIC, SA).

437 *Continued cocirculation of 4c-recombinant lineages*

438 The 4c-recombinant viruses were initially assigned to lineages based on the GI.2 VP60
439 phylogeny (Figure 3A). For GI.4c-recombinant viruses where a full genome sequence
440 was not available, we first inferred a phylogeny from a 504 nt region spanning the
441 RdRp-VP60 junction (Figure 5); this phylogeny was annotated using taxa for which the
442 lineage was definitively known from the VP60 phylogeny. While sequences from each
443 4c lineage (as defined from the complete genome sequences) did not form clades with
444 individual common ancestors in the RdRp-VP60 junction phylogeny, they did form
445 distinct groups with visible genetic distance between them (Figure 5). These groups
446 were distinct enough to assign a 4c lineage type to those samples with partial sequence
447 only. After assigning lineages to all GI.4c-recombinants, we explored the interactions
448 between the different lineages by mapping their sampling locations over time. We found
449 that four of the five lineages were already circulating in 2017 (Figure 5). Lineage ii was
450 not detected after 2017 while the other four lineages continued to be detected
451 throughout 2018, 2019, and 2020. In VIC, where all lineages are circulating, there was
452 no clear indication of geographical clustering of the different lineages. In TAS, lineages
453 ii, iii, and iv were detected sporadically while lineage v has remained dominant over
454 time, suggestive of a founder effect in this region. Similarly, in NSW/ACT lineage iv has
455 remained dominant, also suggesting a regional founder effect, while lineages i, ii, and iii
456 were only detected intermittently.

457

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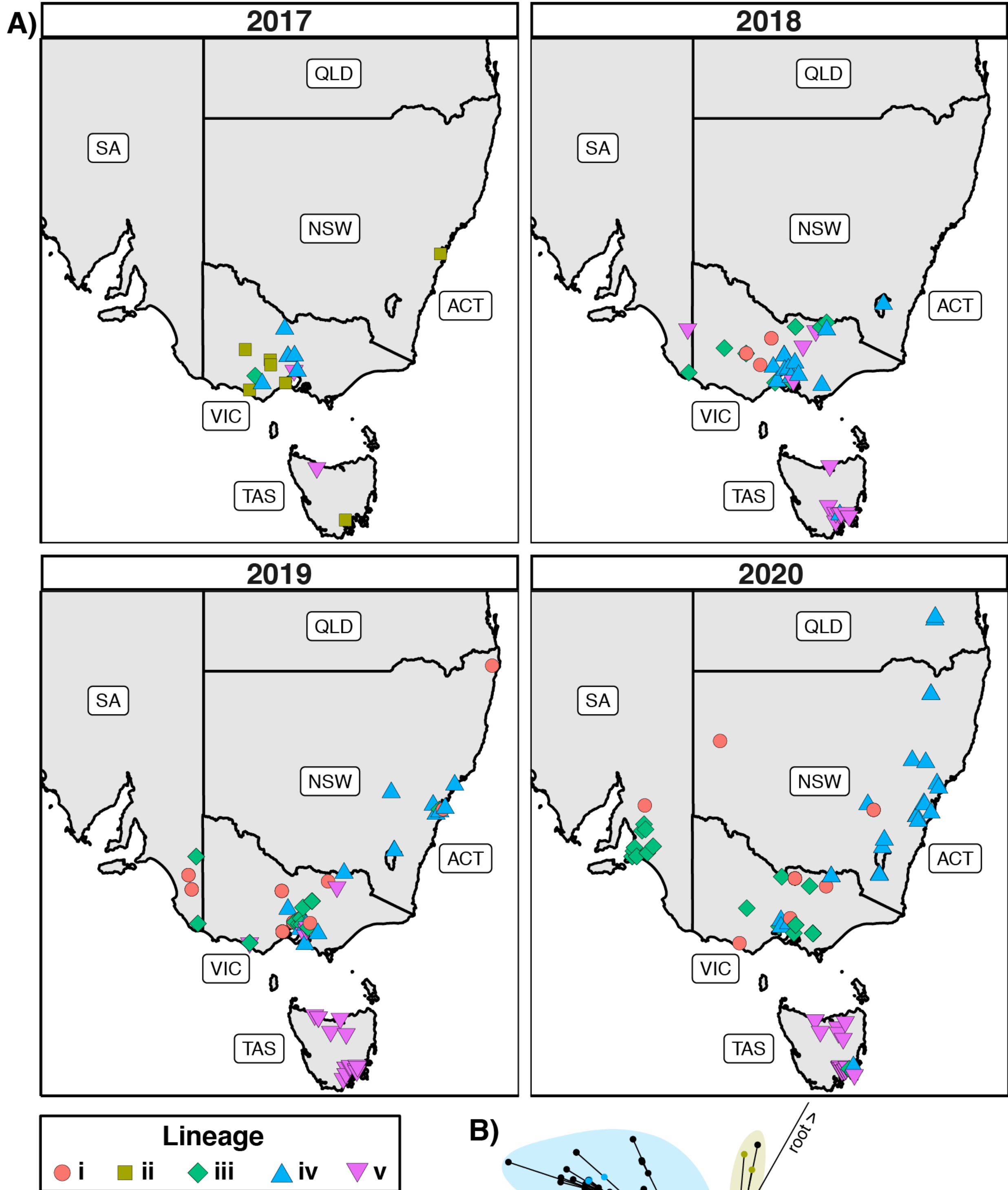

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459 **Figure 5**: **Geographical distribution of 4c-recombinant lagoviruses in Australia,**
460 **2017 to 2020.** (A) Sampling locations for the 4c lineages were mapped for each year
461 following the emergence of this variant in 2017. (B) 4c-recombinant viruses were
462 allocated to a 4c lineage based on ML phylogenetic grouping of the partial RdRp-VP60
463 sequence (504 nt) with reference sequences. Reference sequences were annotated
464 based on full genome sequencing and are indicated in the tree by the coloured tip
465 points. The phylogeny was midpoint rooted and the scale bar represents nucleotide
466 substitutions per site.

467

468 *The emergence of 4c-recombinant variants was not associated with antigenic changes*
469 *in the VP60 capsid protein*

470 To determine whether the observed epidemiological replacement of 1) the parental
471 RHDV2 virus by the novel 4c-recombinant in VIC and TAS, and 2) the 4e-recombinant
472 in NSW/ACT were associated with nonsynonymous, potentially antigenic, changes in
473 the VP60 capsid protein we performed ancestral state reconstruction using our prior
474 Australian GI.2 VP60 phylogeny. Notably, four of the five 4c-recombinant lineages
475 showed no amino acid changes in the capsid protein relative to the inferred ancestral
476 RHDV2 node, suggesting that the emergence of these lineages was not associated with
477 changes in antigenicity (Figure 3A). However, nonsynonymous mutations were
478 identified in 4c-recombinant lineage i and in 4e-recombinant viruses (Figure 3A). When
479 we mapped these mutations back to an atomic model of lagovirus GI VP60, both
480 mutations were within the protruding domain, specifically the P2 subdomain, which is
481 known to contain immunodominant epitopes (Figure 3B) [61]. The 4c-recombinant
482 lineage i was associated with an S364G mutation, while an I434T mutation was present
483 in all lineage 4e-recombinants (Figure 3A). Thus, it is possible that the epidemiological
484 fitness of the 4e-recombinant variant in NSW/ACT may be attributable to antigenic
485 changes in the capsid; however, this cannot explain the replacement of parental RHDV2
486 by 4c-recombinant variants in VIC or TAS, or the continued co-circulation of 4c-
487 recombinant lineages without changes in VP60. Furthermore, in NSW/ACT the 4e-

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488 recombinant variant, despite the I434T change in VP60, appears to be currently
489 undergoing replacement by the 4c-recombinant.

490

491 ## Discussion

492 In a global landscape where population immunity to GI.1 and GI.1a viruses was
493 widespread in lagomorphs, it is unsurprising that GI.2 viruses were so successful and
494 rapidly replaced GI.1 strains. We were interested to determine whether, after
495 establishment, GI.2 viruses would continue to evolve via immune selection and
496 antigenic drift or by other mechanisms. We were also interested to document whether
497 variants without the GI.2 capsid would emerge as population immunity increased
498 against this genotype. Instead, we found that the GI.2 capsid was retained and that the
499 emerging recombinant variants acquired alternative NS genes. It is becoming
500 increasingly clear that recombination is a major driver of calicivirus diversity, facilitating
501 the emergence of new variants including those with pandemic and panzootic potential
502 [24, 25, 62, 63]. However, the frequency at which viable recombination occurs and the
503 genetic drivers of epidemiological fitness are still poorly understood. In this study, a
504 continent-scale natural competition experiment of lagoviruses in Australia, we found that
505 viable recombination occurs at an extremely high frequency given the right agent, host,
506 and environmental circumstances.

507 *Six viable recombination events in Australian lagoviruses between 2014 and 2018*

508 Shortly after detecting two exotic lagovirus incursions in Australia, RHDVa-Aus (GI.4eP-
509 GI.1a) in 2014 and RHDV2 (GI.1bP-GI.2) in 2015 [32, 37], we reported the emergence
510 of a recombinant variant of these two viruses (4e-recombinant; GI.4eP-GI.2), detected
511 in July 2016 in NSW [31]. This finding prompted the development of improved molecular
512 tests for the detection of recombination and the subsequent retrospective and
513 prospective screening of lagovirus-positive samples for further recombination events.
514 This screen identified an additional novel recombinant lagovirus, a GI.4cP-GI.2 variant
515 (4c-recombinant), first detected in VIC in February 2017. Further analysis determined
516 that the VP60 genes of this 4c-recombinant separated into five distinct clades (Figure

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517 3A), suggesting that this variant emerged on five separate occasions following at least
518 five independent recombination events. Recombination was already known to occur in
519 lagoviruses. For example, in the last decade, the lagovirus GI.2 capsid has been
520 detected in combination with five different sets of NS genes [24, 27, 31, 33]. Our study
521 demonstrates that viable recombination during natural infection occurs even more
522 frequently than previously appreciated but may often go undetected if different events
523 result in closely related progeny. Since the first detection of RHDV2 in Australia in mid-
524 2015 [37] this capsid gene has recombined and produced epidemiologically viable
525 variants at least six times in a two-year period.

526 The previously reported 4e-recombinant arose from a recombination event between
527 RHDVa-Aus (GI.1aP-GI.2) and RHDV2 (GI.1bP-GI.2), both recombinant viruses
528 themselves that were exotic incursions detected in Australia in January 2014 and May
529 2015, respectively, although the source of these viruses remains unknown [31, 32, 37].
530 Detections of the parental RHDVa-Aus were predominantly restricted to the Sydney
531 basin region of NSW, with two cases detected in QLD and one further case in a wild
532 rabbit in the ACT [32]. Therefore, it is not surprising that the 4e-recombinant emerged in
533 NSW/ACT, according to our epidemiological data and phylogeographic analysis (Figure
534 4). The 4c-recombinants arose from recombination events between RHDV2 (GI.1bP-
535 GI.1) and endemic RCV-A1 (GI.4c) viruses [14, 37]. Emergence of the 4c-recombinant
536 variants in the south-eastern states of Australia is consistent with high rabbit population
537 densities and a high seroprevalence to GI.4 viruses in these regions compared to more
538 arid regions of Australia [64].

539 Despite the VP60 phylogeny suggesting multiple recombination events between GI.4c
540 and GI.2 viruses, only a single unique recombination event was detected by the RDP4
541 program. This is not surprising given that the breakpoint locations are indistinguishable
542 and these recombinants (and their true parents) are very closely related. Indeed, it was
543 only possible to infer the true number of recombination events because of the high
544 sampling frequency of parental RHDV2 viruses, which has been maintained for this and
545 previous studies. Had RHDV2 sequences from intervening clades not been sampled,
546 the 4c-recombinants would have formed a monophyletic clade and a single

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547 recombination event assumed. This is demonstrated by the NS genes phylogeny
548 (Figure 4A), in which the 4c-recombinant sequences form a single clade within the
549 greater GI.4c clade. Importantly, within this clade the sequences group into the same
550 five lineages as seen in the VP60 tree. Compared to pathogenic GI.2 viruses, benign
551 RCV-A1 (GI.4c) viruses are greatly under-sampled in Australia, with the most recent
552 RCV-A1 sequences (n = 6) derived from samples collected from the ACT in 2012 and
553 2014 [35]. Aside from three sequences sampled in 2014, no other RCV-A1 sequences
554 were available for the entirety of the study period. Consequently, a close relative of the
555 RCV-A1 parental virus has not been sequenced, suggesting a hidden diversity of
556 unsampled RCV-A1 viruses in Australian rabbits. This study provides a lower bound to
557 the rate of recombination in these viruses; it is possible that additional recombination
558 events have occurred with GI.4c viruses that are not observable against the current
559 background of available GI.2 VP60 sequences.

560 *Lagoviruses show frequent recombination between the GI.2 capsid and GI.4 NS*
561 *sequences*

562 Interestingly, all the recombination events detected during the study period involved the
563 pairing of a GI.2 capsid and the NS genes of a GI.4 virus. Despite screening over 800
564 lagovirus-positive samples, we found no evidence for recombinant variants with either
565 capsid or NS sequences of GI.1 viruses.

566 For recombination to occur several criteria must be met [23]. Firstly, there must be
567 coinfection of an individual host. This is influenced by host tropism, the prevalence of
568 each parental virus in the population, and the duration of infection. Secondly, there must
569 be coinfection of a single cell, both viruses must replicate within this cell, and precise
570 template switching must occur to generate viable gRNA. Finally, the resultant variant
571 must be epidemiologically competitive. That is, it must be able to successfully transmit,
572 to establish infection in new hosts, and to avoid being outcompeted by either the
573 parental or other circulating variants.

574 Following the epizootic incursion of an antigenically novel virus, RHDV2, into a naïve
575 population, the prevalence of this variant was extremely high in Australian rabbits,
576 reflected by the estimated population-wide mortality rate of 60% following the arrival of

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577 RHDV2 [39]. The detection of four GI.1 and GI.2 co-infections in this study between
578 2015 and 2017 demonstrates that both viruses were circulating at sufficiently high
579 prevalence for mixed infections to occur. However, this doesn't explain the predilection
580 for GI.4 (RCV) NS sequences. The discovery of 4c-recombinants was surprising, since
581 the parental RCV-A1 is a benign enterotropic lagovirus [14], while RHDV2 (and RHDV1)
582 viruses are virulent and hepatotropic. This would seem to preclude coinfection of
583 individual cells with both viruses. Yet, several recombinants of RHDV2 and benign
584 enterotropic RCVs have also been reported from Europe [64]. This suggests that active
585 replication of both RCVs and RHDV2 must be occurring in the same target cell.
586 Extrapolating from our current understanding of human and murine norovirus tropism,
587 macrophages or other immune cells may be likely candidates [65]. This is further
588 supported by the detection of plus- and minus-strand viral RNAs in splenic and alveolar
589 macrophages of rabbits experimentally infected with RHDV1 [66]. Additionally, RCVs
590 typically infect young rabbits early in life [12, 14]. Although young rabbits can be
591 infected with RHDV1 viruses, robust innate immunity limits the extent of viral replication
592 in an age-dependent manner [34, 39]. Thus, mixed infections with RHDV1 and RCVs
593 are probably infrequent. This age-dependent resistance is not observed with RHDV2
594 infection [67]. Furthermore, the duration of infection is longer for benign RCVs
595 compared to pathogenic variants, where infected individuals typically die within 48 – 72
596 hours post-infection [2]. These factors may at least partly explain why RCV-RHDV2
597 recombinants appear to emerge more frequently than RHDV1-RHDV2 recombinants.

598 *Epidemiological drivers of lagovirus emergence and spread*

599 Both the 4e-recombinant and 4c-recombinant rapidly replaced the dominant circulating
600 parental RHDV2 in NSW/ACT and VIC/TAS, respectively. This replacement, at least for
601 several 4c-recombinant lineages, occurred without any associated antigenic changes in
602 the capsid protein, demonstrating that the replacement was not driven by antigenic
603 escape. This shows that NS sequence variation is an important driver of epidemiological
604 fitness in lagoviruses, complementing similar findings in human and murine noroviruses
605 [68, 69]. For example, the pandemic GII.P16/GII.4 Sydney 2012 norovirus, which does
606 not contain unique substitutions in the capsid, has substitutions within the RdRp that are

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607 proposed to increase transmissibility [69]. Infection with this variant also resulted in
608 increased viral shedding compared to other norovirus genotypes, as measured by
609 higher faecal viral loads [70]. Previous studies have shown that RdRp fidelity and intra-
610 host viral diversity affect transmissibility of murine norovirus *in vivo*, with high-fidelity
611 variants being less efficiently transmitted than the wild-type variant [71]. Taken together,
612 these studies demonstrate that NS proteins, particularly the RdRp, are important drivers
613 of calicivirus fitness. Interestingly, the *in vitro* polymerase replication activity of the
614 cloned GI.4c RdRp was previously shown to be at least two times that of the RHDV1
615 (GI.1c) RdRp [72], although comparison to the GI.1b RdRp, the specific 'competing'
616 variant in the current study, was not reported. Analogous to the findings in murine
617 norovirus, we propose that a higher replication rate of GI.4 RdRps may lead to
618 increased intra-host viral diversity and transmissibility of GI.4 recombinants. However,
619 we cannot rule out that other NS proteins may also contribute to the observed high
620 fitness of the recombinant variants.

621 Within the 4c-recombinants, there was no evidence of dominance of any one lineage
622 over time. The apparent dominance of lineage iv in NSW/ACT and lineage v in TAS is
623 consistent with founder effects in both of those states. This demonstrates that different
624 lagoviruses can cocirculate at relative equilibrium over extended periods of time. This
625 further supports that the observed rapid replacement of parental RHDV2 (GI.1bP-GI.2)
626 by GI.4P recombinant variants in this study is due to a fitness advantage of these
627 variants, conferred by the NS proteins and possibly associated with the RdRp.

628 *The lagovirus capsid governs host and tissue tropism and is correlated with virulence*

629 Both the newly identified 4c-recombinant and the previously emerged 4e-recombinant
630 are virulent, hepatotropic viruses that were recovered from the livers of both rabbits and
631 hares and from rabbits of all ages in this study. This tropism mimics that seen with other
632 GI.2 viruses [73-80]. In stark contrast, the parental RCV-A1 is a benign, enterotropic
633 virus that has only been recovered from rabbits [14], while the RHDVa-Aus variant,
634 although virulent and hepatotropic, has only been found in adult rabbits. Our findings
635 suggest that it is the lagovirus capsid that confers both host and tissue tropism and that
636 tissue tropism is correlated with virulence.

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637 With the broader host tropism conferred by the GI.2 capsid, there is increased potential
638 for the emergence of novel epizootic lagovirus variants through both intragenotypic and
639 intergenotypic recombination. Hares are known to carry their own, presumed benign,
640 caliciviruses [16-18, 31, 33, 73-79]. Indeed, the first intergenotypic lagovirus
641 recombinants were recently reported from Germany [33]. Since the incursion of RHDV2
642 into North America in 2020 this variant has also been reported to infect several
643 *Sylvilagus* species (cottontail rabbits) [81]. Although endemic *Sylvilagus* caliciviruses
644 have never been reported, very limited sampling has been conducted in this species. It
645 remains to be seen whether North American leporids may be a new reservoir for the
646 emergence of novel lagoviruses with panzootic potential. This highlights the need for
647 ongoing surveillance and full genetic characterization of lagoviruses and other
648 caliciviruses to facilitate detection of future emerging variants of significance to both
649 animal and human health.

650

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671

672 **Data availability**

673 Full genome sequences are available in GenBank under accession numbers
674 MW460205 – MW460242. All sequence alignments, tree files, and BEAST xml files are
675 available at https://doi.org/10.25919/758f-4t15.

676

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