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Evolution and Phylogeography of the Nonpathogenic Calicivirus RCV-A1 in Wild Rabbits in Australia▽

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Despite its potential importance for the biological control of European rabbits, relatively little is known about the evolution and molecular epidemiology of rabbit calicivirus Australia 1 (RCV-A1). To address this issue we undertook an extensive evolutionary analysis of 36 RCV-A1 samples collected from wild rabbit populations in southeast Australia between 2007 and 2009. Based on phylogenetic analysis of the entire capsid sequence, six clades of RCV-A1 were defined, each exhibiting strong population subdivision. Strikingly, our estimates of the time to the most recent common ancestor of RCV-A1 coincide with the introduction of rabbits to Australia in the mid-19th century. Subsequent divergence events visible in the RCV-A1 phylogenies likely reflect key moments in the history of the European rabbit in Australia, most notably the bottlenecks in rabbit populations induced by the two viral biocontrol agents used on the Australian continent, myxoma virus and rabbit hemorrhagic disease virus (RHDV). RCV-A1 strains therefore exhibit strong phylogeographic separation and may constitute a useful tool to study recent host population dynamics and migration patterns, which in turn could be used to monitor rabbit control in Australia.

Lagoviruses form a genus within the *Caliciviridae* family of RNA viruses (16). All representatives of this genus are highly species specific and infect only their respective hosts, i.e., rabbits and hares. The prototype species of the genus *Lagovirus* is rabbit hemorrhagic disease virus (RHDV), which was first described in China in 1984 when a severe infectious necrotizing hepatitis with mortality rates up to 90% was observed in angora rabbits (22). However, phylogenetic analysis indicates that the pathogenic strains of RHDV likely evolved from nonpathogenic lagoviruses several decades before they were initially described (20). Today, RHDV is found on most continents, causing ongoing damage to the rabbit meat industry (24) and threatening wild native rabbit populations in Europe. On the Iberian peninsula the rabbit is considered an endangered species and is itself a staple food of endangered predators, such as the imperial eagle and the Iberian lynx (10).

In marked contrast, since 1995 Australia has been using RHDV as a successful viral biocontrol agent for rabbits (7), which cause severe environmental and economic damage in this country. The use of a viral biocontrol agent for a vertebrate species has been and remains controversial, but there is little doubt that since its release RHDV has generated close to $6 billion of savings to the Australian agricultural industry (41), as well as some much needed relief for the regeneration of many native plant species (35).

Notably, RHDV-induced mortality is lower in certain areas of Australia, namely, the cooler and more humid southeast region of the continent, which is believed to be in part due to the presence of related but nonpathogenic lagoviruses circulating in the population (8). Recently, such a virus was identified in Australian wild rabbits (38). This new member of the genus *Lagovirus*, termed rabbit calicivirus Australia 1 (RCV-A1), is a nonpathogenic virus causing a predominantly enteric infection in rabbits. Other benign or moderately pathogenic RCV strains have also been described in the United States and Europe (1, 5, 14, 15). While the Italian nonpathogenic RCV provides complete cross-immunity to RHDV, only partial protection is conveyed by the Australian virus RCV-A1 (37) although this may be sufficient to reduce overall RHDV-induced mortality.

Like all lagoviruses, RCV-A1 has a single-stranded positive-sense RNA genome of approximately 7.5 kb that is polyadenylated and has a viral protein (VpG) covalently bound to its 5′ end (25). The genome is organized into two open reading frames (ORFs) (6, 39). ORF1, which represents the majority (7 kb) of the genome, encodes a polyprotein that is auto-proteolytically cleaved during posttranslational processing into several smaller proteins, including the helicase, protease, and polymerase (26). In contrast, ORF2 is only 351 nucleotides (nt) long, and the function of its VP10 gene product is unknown although it is present in small amounts in the virion (39).

Given the potential interactions between RCVs and pathogenic RHDV, which could impact rabbit survival in both Australia and Europe, it is clearly important to understand the evolutionary history, genetic diversity, and geographic distribution of RCV-A1 and other benign lagoviruses. In Australia, growing reports of rising rabbit numbers mean that there is a clear need for improved strategies to monitor and control rabbit populations. In addition, because RCV-A1 does not cause any fitness decrease in its rabbit host (37), it is possible that this virus could be used as a population genetic marker to

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TABLE 1. Field sites where RCV-A1 was present including the samples used in analysis

Region[a]	Site	Year	Location	Sample	Sequenced region (nt)[b]	GenBank accession no.
Canberra, ACT	Gungahlin	2007	Lat −35.13, long 149.07	GUN 1-11	4566–7159	GU368903
Bathurst, NSW	Valpine	2007	Lat −33.23, long 149.28	V-5	4458–7162	GU368899
				V-8	4458–7162	GU368900
				V-11	4566–7159	GU373616
	Oakey Creek	2007	Lat −33.20, long 149.20	OC-7	4458–7162	GU368898
				OC-13	4458–7162	GU368894
				OC-15	4458–7162	GU368895
				OC-20	4566–7159	GU368908
				OC-21	4566–7159	GU368909
				OC-26	4458–7162	GU368896
				OC-33	4566–7159	GU368910
				OC-36	4458–7162	GU368897
				OC-39	4566–7159	GU368911
				OC-40	4566–7159	GU368912
Hawkesbury, NSW	Cattai National Park	2007	Lat −33.32, long 150.30	CAT 2-5	4566–7159	GU368905
				CAT 2-10	4566–7159	GU368904
				CAT 2-12	4458–7162	GU368889
				CAT 3-4	4458–7162	GU368890
				CAT 7-1	4458–7162	GU368891
Michelago, NSW	Michelago	2007	Lat −35.44, long 149.09	MIC 1-5	4566–7109	GU373614
				MIC 3-3	4458–7162	GU368892
				MIC 4-6	4566–7159	GU368907
				MIC 4-9	5290–7032	GU373615
				MIC 5-8	4566–7159	GU368893
				MIC 5-10	4458–7162	GU368906
				RCV-A1 Michelago	Complete genome	EU871528
Burragate, NSW	Burragate	2007	Lat −37.00, long 149.37	BUR 1-1	4458–7162	GU368888
Bacchus Marsh, VIC	Bacchus Marsh	2009	Lat −37.39, long 144.20	BM-40	4566–7159	GU368916
				BM-41	4566–7159	GU368917
				BM-49	4566–7159	GU368919
				BM-58	4566–7159	GU368918
Bendigo, VIC	Bendigo	2009	Lat −37.00, long 144.22	BEN-12	4566–7159	GU368913
				BEN-16	4566–7159	GU368914
				BEN-26	4566–7159	GU368915
				BEN-35	4458–7162	GU368902
Wauchope, NSW	Wauchope rabbitry	2009		WAU-1	4458–7162	GU368901

[a] ACT, Australian Capital Territory; NSW, New South Wales, VIC, Victoria.
[b] Nucleotide positions are numbered in reference to the previously published RCV-A1 Michelago sequence.

track movements and changes in the distribution of the European rabbit across the Australian continent. Indeed, other microorganisms have proven to be useful indicators of the population dynamics of their hosts (3, 42). Microbial markers are particularly informative for the study of population processes in the very recent past since genetic changes will not have had sufficient time to be recorded in the more slowly evolving host genome (2).

The host-pathogen interaction of Australian rabbits and their viruses is unique in that both were introduced only once (or possibly a few times in a limited period of time). In addition, 150 years of historical records are available documenting the introduction, spread, and control efforts of rabbits in Australia. Herein, we explore the evolutionary history and dynamics of RCV-A1 in Australia, with a particular focus on revealing the phylogeographic distribution of the virus across this continent, the evolutionary processes that have shaped its diversity, and its suitability as a marker to study host distribution and migration patterns.

MATERIALS AND METHODS

Sampling. Eight sites with a high occurrence of wild rabbits were sampled in southeast Australia, mainly during the breeding season from 2007 to 2009 (Table 1 and Fig. 1). All sites were reported to have low biocontrol success and an average annual rainfall of more than 600 mm. Rabbits were caught alive in cage traps or with ferrets. The cages were set approximately 1m from an active entrance to a warren, facing toward it, or in areas where rabbits were foraging. Traps were baited with diced carrots and usually not prebaited. When ferrets were used, warren entrances were covered with purse nets, and a ferret was released into the warren to flush out the rabbits. Caught animals were killed by cervical dislocation, weighed, and sexed; blood was collected by cardiac puncture, and tissue samples were taken. Tissue samples were immediately frozen at −20°C. In addition, samples from domestic rabbits from one rabbitry were collected. The methods described above were approved by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Sustainable Ecosystems Animal Ethics Committee (SEAEC

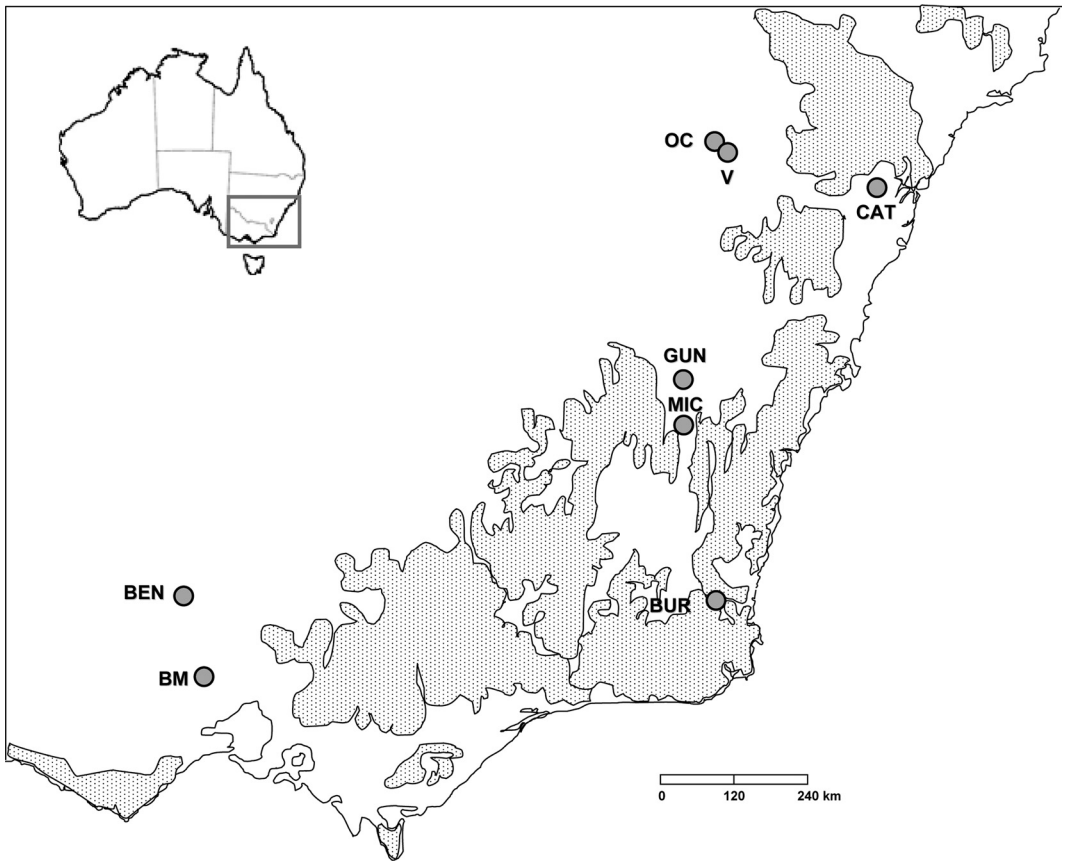

FIG. 1. Map of sampling locations of RCA-A1 in southeast Australia. Forested areas in the investigated area of the Great Dividing Range are shaded. See Table 1 for site identifications.

06-31) using the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Extraction, detection, and sequencing of viral RNA. Viral RNA was extracted from 50 mg of duodenum using Trizol (Invitrogen, Mulgrave, Australia) according to the manufacturer's instructions. Reverse transcription (RT) was carried out with oligo$(dT)_{20}$ primer and Superscript RT III (Invitrogen) according to the protocol suggested by the supplier. Samples collected in 2007 were screened using standard PCR and the primer set Rab1b/Rab2 as described previously (38). For samples collected in 2009, a one-step real-time PCR was developed as a sensitive and more rapid detection tool for RCV-A1. The oligonucleotides RCV realtime fw and RCV realtime rev2 (Table 2) were designed to be relatively specific to RCV-A1 and to amplify a fragment of 160 nt. A QuantiTect SYBR Green RT-PCR Kit (Qiagen) was used according to protocols suggested by the supplier. Initial screening was conducted on pooled samples with a pool size of five. Samples from positive pools were retested individually. RNA from positive samples was reverse transcribed, and the presence of RCV-A1 was confirmed by standard PCR and by sequencing.

PCR amplifications were performed with specific and degenerate oligonucleotides in 20- or 50-μl PCR mixtures. Degenerate oligonucleotides were designed to bind in regions of sequence conservation between lagoviruses or RCV-A1 strains. Specific oligonucleotides were designed based on RCV-A1 Michelago (38) and later on sequences defined in this study. The same oligonucleotides used for amplification were subsequently used for direct sequencing of the amplicons at the Australian Genome Research Facility (Brisbane, Australia). Sequences were assembled using BioEdit, version 7.0.1 (17).

Sequence analysis. A total of 67 capsid gene sequences (alignment length of 1,740 nt) comprising viruses currently classified as RCV ($n = 38$) and RHDV ($n = 29$) were used for evolutionary analysis. Of these, 36 represent RCV-A1 from Australia. Multiple sequence alignments were generated with the CLUSTALW program (40) and adjusted manually using BioEdit, version 7.0.1.

Timescale of RCV evolution. The time to the most recent common ancestor (TMRCA) of the combined RCV and RHDV data set was estimated using the Bayesian Markov chain Monte Carlo (MCMC) approach available in the BEAST package (http://beast.bio.ed.ac.uk) (11). Because of the very narrow time range over which RCV-A1 samples were collected, spanning only 2007 to 2009, we were unable to obtain a reliable estimate for the rate of nucleotide substitution and TMRCA from these data alone. A mean substitution rate of 7.7×10^{-4} nucleotide substitutions per site per year previously estimated for RCV and RHDV combined (20) on the basis of sequences covering a far broader span of sampling times was therefore employed as an empirical prior distribution in this analysis. With this rate in hand we were able to estimate the timescale of rabbit lagovirus evolution in Australia.

Two data sets were investigated separately but using identical parameters; the first comprised the 36 RCV-A1 sequences while the second included 67 different sequences from RCV-A1, RCV, and RHDV (as described above). The Bayesian skyline tree prior and the uncorrelated lognormal relaxed molecular clock (12) were applied in all cases. All analyses utilized the general time-reversible (GTR) model of nucleotide substitution with a different substitution rate for each codon position although very similar TMRCA estimates were obtained under an HKY85+Γ_4 substitution model, a strict molecular clock, and a constant populations size tree prior (data not shown; available from the authors on request). All chains were run for 50 million generations, and a stationary solution was achieved in all cases. Statistical uncertainty in each estimate was provided by values of the 95% highest probability density (HPD). Finally, this analysis also allowed us to estimate the maximum clade credibility (MCC) tree for each data set, with Bayesian posterior probability (BPP) values indicating the degree of support for each node.

Phylogeographic analysis. To assess the extent and pattern of phylogeographic structure in the RCV-A1 data, we examined the posterior set of trees generated by BEAST using the BaTS program (28). BaTS calculates the parsimony score (PS) and association index (AI) statistics as a means to quantify the strength of phylogeny-trait associations in the data (with traits representing place of sampling) and the monophyletic clade (MC) size statistic as a measure of how often the sequences cluster together according to their geographic location (28). This

TABLE 2. Oligonucleotides used for the amplification and sequencing of RCV-A1[a]

Primer name	Sequence 5′–3′	Direction[b]	Binding site[c]	Reference or source
RCV realtime rev	GGTGGACCRCCAATYCCCGCCGTTGC	R	5440–5465	This study
Rab1c	GCIGGIACTGCYACCACAGCATCAGT	F	5329–5354	This study
RCV realtime fw	GTTGGYAGGAAYGTRCCCATCATGTTTGC	F	6655–6683	This study
RCV realtime rev2	GTRAGYGCMGACGAGTAATTRTTTAGCGACA	R	6785–6815	This study
RCV-1	GCCAAATGTATGCCGGCTGGGC	F	5609–5630	This study
RCV-2	GTCAAATGTATGCTGGTTGGGC	F	5609–5630	This study
RCV-3rev	TTAACTGCCAAACGAAAAACTGTCCAGG	R	6817–6844	This study
RCV-4	TCAARATGACAGACATYGGTTGGGT	F	4964–4988	This study
RCV-5rev	GTTAGGTAGRTARCCAACAATKGTG	R	1158–1182	This study
RCV-6	CAGYTAYCCACACTTGYTRGACATG	F	2091–2115	This study
RCV-7rev	GTCYTCATCAGGTCTYARCTGCCT	R	3175–3198	This study
RCV-8	CTATCGTGGYATCACTGCMAACAG	F	3780–3803	This study
Lago2	ATGCCTTTTACATCRGTCATAAACTCC	F	4675–4701	This study
Lago5rev	CCWGGRTCRCCDGTTGGGTGGTAC	R	5820–5843	This study
Lago7	GGNCCCTTYGYTCCWGGNAAGAAGA	F	4126–4150	This study
Lago9	TGGNCCNATYGCAGTYGGVRTTGACATGAC	F	4401–4430	This study
Lago11rev	GTCCATACCGTCRGTBGTGGTTCCG	R	5358–5382	This study
recfragfw	CTTCTTGTGCCTRGAYTACTCAAAGTGG	F	4482–4509	This study
recfragrev	CGAACCATYACRAACTCAAAGTCCTCACTTGG	R	5938–5969	This study
recfragrev2	GGTGTRTAYGTRGTGGCAGCAGG	R	6583–6605	This study
Rab2b	GGARTGYTGRGCRGTGTACAGTATGC	R	5552–5577	This study
Rab6	GGYTGGGCTGGTGGCATGCAG	F	5623–5643	This study
MICV-14rev	CTCTTAACTTCATTTGGATTAAAACCTAACC	R	7158–7188	This study
MICV-15	GAGTTCTTTGACTTCATCAAACCAGAGC	F	5143–5170	This study
MICV-16rev	GTCCATACCGTCGGTTGTGGTTCCG	R	5358–5382	This study
MICV-17	CAAACTGGATAAGGTTGATGAGTTC	F	3984–4008	This study
MICV-r1	GGGTGTGTACGTGGTGGCAGCAGGAC	R	6581–6606	This study
RCV-A1 capsid fw	AAAAGCTTATGGAGGGCAAGGCCCGTGCAACG	F	5282–5313	This study
CzE-6rev	AGYTTKGGYTCTTGTTGGTACACCTG	R	3964–3989	This study
Rab1b	CAGCDSGCACTGCYACCACAGCATC	F	5327–5351	38
Rab2	GAAKCKRAACTGCATGCCACCAGCCCA	R	5626–5652	38
MICV-12	GTGAAAGTTATGGCGGTTTTATCG	F	1–24	38
MICV-3	CATTCGAGGACTCCGTTCCAACAGGCC	F	1091–1117	38
MICV-5	GTTATTAGACTGGCAATTGACATTCTGG	F	4531–4558	38
MICV-6rev	GTTGCAGCCCTACTATCTGACCATTCCACC	R	6056–6085	38
MICV-7	GTTGTTGCCAAATCCATCTATGGTGTTGC	F	6484–6512	38
ALR-1	AGTGTTTACAACAACCTDATCAACC	F	5866–5890	38
ALR-6rev	CTCGCCAGTGGTATTATAAATCTTAACAC	R	7326–7354	38
CzE-1	GGKARGCAGTGGGCAAAGAAGGTTGT	F	754–779	38
CzE-2rev	GTTGTGAGCTTGCCAGCRCCCTTCATG	R	1203–1229	38
CzE-4rev	CCATACATMASAAAGTACTGTTTCCAC	R	2208–2234	38
CzE-7	CWAAACACCTKTACAAGTGYTGGAG	F	4190–4214	38
CzE-8rev	AACKGCTGCRGACCAAAGCAACCAATGAC	R	4706–4734	38

[a] Several overlapping fragments were amplified and sequenced to obtain the entire capsid region.
[b] F, forward; R, reverse.
[c] Binding positions are given in relation to the genome of RCV-A1 Michelago.

analysis utilized 1,000 randomizations, with the first 10% of trees removed as burn-in. The extent of geographic structuring was investigated (i) for each sampling location (eight populations) and (ii) for sampling locations west of and east of/within the Great Dividing Range (two populations). Only RCV-A1 isolates from wild rabbits were included in this analysis; strain WAU-1 isolated from a commercial rabbitry was excluded.

Selection pressures in RCV-A1. Four different codon-based maximum-likelihood (ML) methods within the online version of the HyPhy package available at the Datamonkey website (http://www.datamonkey.org) (30) were used to estimate the ratio of nonsynonymous to synonymous nucleotide substitutions per site (*dN*/*dS*), itself a measure of selection pressure. Specifically, the methods of single-likelihood ancestor counting (SLAC), fixed-effects likelihood (FEL), and random-effects likelihood (REL) (21) were used to infer selection pressures at individual codons in the RCV-A1 alignment, while the method partitioning approach for robust inference of selection (PARRIS) (36) was used to investigate the possibility of positive selection (*dN*/*dS* of > 1) across the entire capsid region. In each case the confidence level was set to a *P* value of 0.05 and a Bayes factor of 50.

Recombination in RCV-A1. We used both the genetic algorithms for recombination detection (GARD) and RDP3 (recombination detection program) (19) programs to determine whether recombination had occurred in the evolutionary history of RCV-A1. Two different data sets were analyzed, reflecting the fact that the sequence region between capsid and polymerase was previously reported to be a likely recombination breakpoint in the *Caliciviridae* (4) (Table 1). The first data set included the capsid gene plus 833 nt upstream (nucleotide positions 4566 to 7159) from 31 sequences of RCV-A1 while the second included the capsid gene plus 725 nt upstream of the capsid (nucleotide positions 4458 to 7162) from 16 RCV-A1 sequences.

Nucleotide sequence accession numbers. All sequences generated in the course of this study have been deposited in GenBank under the accession numbers listed in Table 1.

RESULTS

Presence of RCV-A1 in southeast Australia. RCV-A1 was present and confirmed by gene sequencing in 44 wild rabbits from eight sites and in one domestic rabbit from a rabbitry. The entire capsid region from 36 samples was sequenced; the viral RNA concentration in the remaining eight samples was

too low to allow successful amplification of more than the diagnostic fragment. Of these samples, an additional 833 nt from 16 samples and an additional 725 nt from 19 samples upstream of the capsid sequence were obtained for recombination analysis (Table 1).

Evolutionary relationships among RCV-A1. Our Bayesian phylogenetic analysis revealed that all Australian RCV strains form a monophyletic group that can be subdivided into six clades (Fig. 2). Nodes are well supported in each case (BPP values of >0.99). As such, it is clear that the Australian RCV-A1 strains form a separate lineage among the caliciviruses described in rabbits. In addition, all pathogenic RHDVs form a clade, which shows a subdivision between the "prototype" RHDV isolates and the antigenic variant subtype RHDVa. More notably, the nonpathogenic European RCVs are more closely related to RHDV than to RCV-A1 so that viruses assigned to the RCV species do not form a monophyletic group. Although such a mismatch between phylogenetic relationship and current species assignment means that a taxonomic revision is required, more broadly our analysis indicates that there is a large assemblage of caliciviruses that infect rabbits and that these viruses can differ markedly in pathogenicity.

The timescale of RCV-A1 evolution. Because of the very limited timescale of sampling of RCV-A1, we used the mean evolutionary rate of 7.7×10^{-4} substitutions/site/year for previously estimated for RHDV and RCV combined (20) as an empirical prior distribution on the substitution rate; this enabled us to estimate a TMRCA for RCV-A1 in Australia. The posterior distribution of substitution rates generated by this analysis was as follows: 7.4×10^{-4} substitutions/site/year (95% HPD, 6.0×10^{-4} to 8.6×10^{-4} substitutions/site/year) for RCV-A1 in isolation and 6.6×10^{-4} substitutions/site/year for RCV and RHDV combined (95% HPD, 5.4×10^{-4} to 7.8×10^{-4} substitutions/site/year); as expected, these values were close to the prior values used. Although there is clearly the potential for error when a substitution rate is used that is estimated from sequences different from those obtained here, for the RCV-A1 rate to be radically different would require major differences in either background mutation rates, replication times, or selection pressures among the assemblage of rabbit caliciviruses. This seems unlikely, given their close phylogenetic relationship. In addition, most fixed mutations in RCV-A1 are likely to be neutral (see below), such that a history of population bottlenecks will not affect substitution rates.

Under these rates, the TMRCA of RHDV and RCV dates back to before the end of the 18th century, although with a large statistical uncertainty (95% HPD, AD 1462 to 1783) (Fig. 2). Hence, this analysis suggests that the Australian RCV-A1 and RCV/RHDV lineages diverged before the introduction of rabbits onto the Australian continent. Similarly, our molecular clock estimates suggest that the RCV-A1 isolates sampled here share an ancestor that likely existed at some point during the 19th century (95% HPD, AD 1771 to 1908) (Fig. 2). These dates are in strong accordance with the introduction of wild European rabbits into Australia in 1859, such that RCV-A1 may have arrived in Australia with these first wild rabbits.

Two later RCV-A1 divergence events are also recorded in these sequence data (Fig. 2). First, the common ancestors of clades 3 and 4 (95% HPD, AD 1915 to 1975) and 5 and 6 (95% HDP, AD 1911 to 1976) seem to have circulated at approximately the same time period during the 20th century while the estimated coalescence time of clades 1 and 2 (95% HPD, AD 1936 to 1987) encompasses the 1950s to 1960s. These estimates overlap with the introduction of the viral biocontrol agent myxoma virus into Australia in 1950/1951 and the subsequent collapse of the rabbit population. It is therefore possible that this major demographic event had a noticeable impact on the genetic diversity of RCV-A1. Second, many of the coalescent events within each of the individual clades date to the 1990s and hence coincide with the introduction of RHDV as a second viral biocontrol agent. RHDV also led to a major crash in the rabbit population over most of the Australian continent.

Such major reductions in population size are expected to increase the strength of genetic drift in viral populations. Indeed, we found only relatively weak evidence for positive selection in RCV-A1. Specifically, all four ML methods employed here confirmed that the vast majority of codon sites are subject to negative selection (dN/dS of < 1) or evolve neutrally although it is important to note that all four methods lack the power to detect adaptive evolution that has occurred on single nucleotide sites on individual viral lineages. Only three sites were identified as possibly subject to positive selection by either the SLAC, FEL, or REL method–amino acid positions 76, 307, and 456—although inconsistent results were obtained among methods, and the biological significance of these sites is uncertain.

Geographic distribution of RCV-A1 strains. Most of the RCV-A1 sequences cluster according to their place of sampling although sample numbers at some sites, namely, Burragate (BUR) and Gungahlin (GUN), were too low to provide a meaningful representation. A mixture of two RCV-A1 clades was found in the rabbit populations at Cattai National Park ([CAT] clades 1 and 3) and in Michelago ([MIC] clades 2 and 4), but representatives of only a single clade were found at the majority of sampling sites (Fig. 2). Members of clade 5 were exclusively sampled southwest of the Great Dividing Range (Bendigo [BEN] and Bacchus Marsh [BM]), clade 6 viruses were found in the central tablelands west (Oakey Creek [OC] and Valpine [V]), and clade 1 viruses were found east of the Great Dividing Range (at CAT).

The strong geographical subdivision of the RCV-A1 populations was confirmed by phylogeny-trait association analysis; the AI and PS statistics, which measure the overall level of population subdivision, were strongly significant ($P < 0.005$), as were the MC statistics for six of the eight virus populations ($P < 0.005$). The two RCV-A1 populations that did not show significant P values under the MC statistic (GUN and BUR) possess only a single sequence each. In addition, the forested areas of the Great Dividing Range were identified as a major landscape boundary associated with population subdivision among the RCV-A1 strains; the AI, PS, and MC statistics strongly supported ($P < 0.005$) a subdivision between populations located west of or east of/within the Great Dividing Range (Fig. 1).

Recombination in RCV-A1. The GARD and RDP3 programs identified CAT 3-4 as a possible recombinant of WAU-1 and an unknown parental strain although the resulting phylogenetic incongruities were minor. The putative recombination

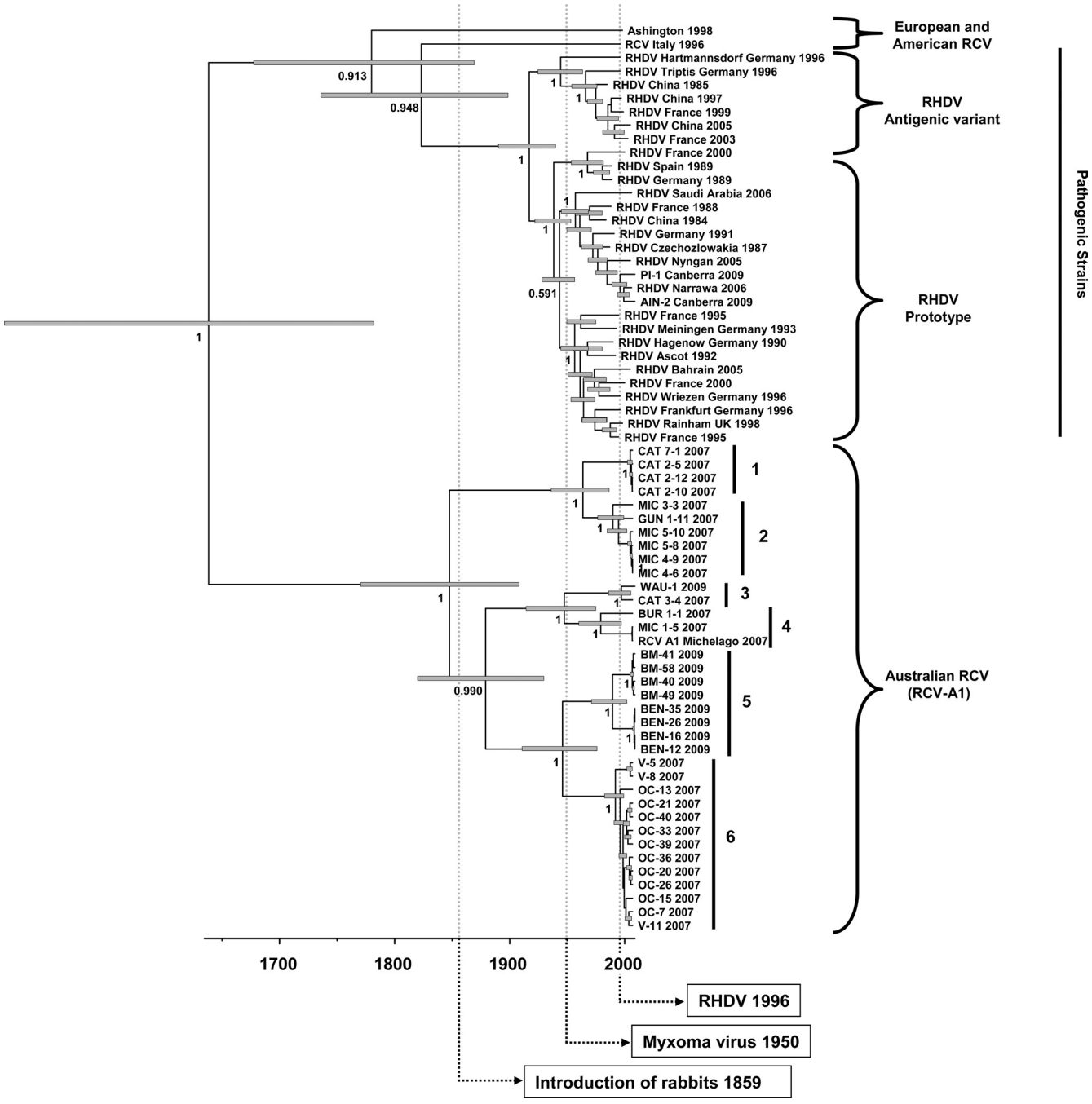

FIG. 2. Bayesian MCC tree of RCV-A1 and RHDV sequences. A timescale (years) is provided on the *x* axis. The node bars depict 95% HPD values on node height (age). Bayesian posterior probability values are shown for major nodes. In all cases tip times reflect the year of sampling. Numbers 1 to 6 depict the different clades of RCV-A1. The times of introduction of rabbits, myxoma virus, and RHDV into Australia are indicated below the *x* axis. GenBank accession numbers of the RHDV strains used are as follows: RHDV China 1984, accession number AF402614; RHDV Rainham United Kingdom 1998, AJ006019; RHDV France 1999, AJ302016; RHDV France 2000, AJ319594; RHDV France 2000, AJ495856; RHDV France 1995, AJ535092; RHDV France 1995, AJ535094; RHDV France 2003, AJ969628; RHDV China 1985, AY269825; RHDV China 2005, DQ069280; RHDV Bahrain 2005, DQ189077; RHDV Saudi Arabia 2006, DQ189078; RHDV China 1997, DQ205345; RHDV Ascot United Kingdom 1992, EF558575; RHDV Nyngan AU 2005, EU650679; RHDV Narrawa AU 2006, EU650680; RHDV Ainslie-2 Canberra AU 2009, GU373617; RHDV Pine Island-1 AU 2009, GU373618; RHDV Germany 1989, M67473; RHDV Germany 1991, M67473; RHDV France 1988, U49726; RHDV Czechoslovakia 1987, U54983; RHDV Frankfurt Germany 1996, Y15424; RHDV Hartmannsdorf Germany 1996, Y15425; RHDV Meiningen Germany 1993, Y15426; RHDV Wriezen Germany 1996, Y15427; RHDV Hagenow Germany 1990, Y15441; RHDV Triptis Germany 1996, Y15442; RHDV Spain 1989, Z49271; Ashington United Kingdom 1998, AF454050; and RCV-Italy 1996, X96868. WAU, Wauchope rabbitry.

event is largely located within the highly conserved polymerase region but also includes the first 60 nt of the capsid region (data not shown; available from the authors on request). No other possible recombination events within the capsid genes or between the capsid and the polymerase gene were observed among the RCV-A1 strains analyzed here although as full-length genomes were not compared, it was impossible to determine whether there were potential recombination events upstream of the polymerase gene.

DISCUSSION

Our study reveals that evolutionary and epidemiological patterns in RCV-A1 likely reflect key demographic events in the history of Australian rabbits. Domestic rabbits were brought to Australia with the first European settlers in 1788 and were repeatedly introduced over the following 70 years but failed to establish long-lasting wild populations (34). The first and main introduction of wild rabbits in Australia occurred in 1859, when Thomas Austin introduced 24 wild-type rabbits from the United Kingdom. These founding rabbits multiplied to several thousand within the first 3 years, and the population front advanced up to 100 km per year, extending the range by 2,500 km across the continent in just 50 years (34). Today, wild rabbits inhabit more than two-thirds of the continent and are considered one of the worst invasive pest species in terms of economic and environmental damage (31).

Our molecular clock analyses support the hypothesis that RCV-A1 was introduced with the first wild rabbits in the 1850s (38). Indeed, it is striking that all RCV-A1 strains sampled here have a single common ancestor that dates to a period coincident with the recorded introduction of Thomas Austin's rabbits 150 years ago (Fig. 2). In addition, our analysis suggests that RCV-A1 diversified into a number of geographically distinct lineages within the first 20 years following the introduction of rabbits. This likely reflects the very rapid initial spread of rabbits in southeastern Australia from Geelong over large parts of Victoria, New South Wales, and South Australia during the same period. However, genetic studies indicate that present rabbit populations in the Sydney area may be derived from a second introduction of rabbits or may be of mixed origin (29). This alternative hypothesis of repeated rabbit introductions cannot be entirely excluded by the RCV-A1 data available at present, particularly as the time frames of possible repeated introductions and the spread of Thomas Austin's rabbits overlap.

Following the introduction of the rabbits, the small founder population expanded rapidly during its colonization of the Australian continent. Again, the data presented here support the notion that RCV-A1 was effectively disseminated via this initial dispersal of rabbits, but once a rabbit population had established, the rate of virus migration decreased. Indeed, our phylogeographic analysis revealed strong population subdivision, with this process starting at the end of the 19th century (see below).

Our analysis further suggests that two other major demographic events had a significant impact on the genetic structure of RCV-A1: the introductions of myxoma virus and RHDV as biocontrol agents on the Australian continent in the 1950s and 1990s, respectively. Both viruses reduced rabbit populations by more than 95% in some areas (13, 27), in turn causing major population bottlenecks in RCV-A1. Such bottlenecks would have had a profound effect on viral population genetic structure, facilitating genetic differentiation through strong genetic drift.

It is also noteworthy that the RCV-A1 strains sampled between 2007 and 2009 show a strong population subdivision at almost all sampling locations. Forested areas of the Great Dividing Range were identified as one major landscape boundary that, according to our molecular clock dates, may have separated RCV-A1 populations since the end of the 19th century. Previous studies have shown that forests strongly limit connectivity of rabbit populations (18) as they do not represent the rabbit's preferred habitat. Our finer-scale phylogeographic analysis reveals that even sequences sampled in rabbit populations less than 20 km apart (Fig. 1, V and OC) show a significant population subdivision, maintained over approximately 15 years (Fig. 2). This relatively small distance may therefore represent a sufficient (although not necessarily absolute) barrier to RCV-A1 spread and is in agreement with early studies based on allozymes showing evidence for spatial structuring of rabbit populations at the 3- to 10-km scale in temperate Australia (33). Although many aspects of RCV-A1 epidemiology are still unclear, it is feasible that the virus persists in isolated rabbit populations by continually reinfecting a proportion of a high-density population where it is endemic, as previously demonstrated for feline calicivirus (9). Whatever the mechanism of persistence in rabbit populations, in terms of transmission RCV-A1 clearly differs from its close relative RHDV, where epidemiologically important long-distance spread via mechanical insect transmission (23) provides regular opportunities for admixture.

Interestingly, in two rabbit populations we observed two different variants of RCV-A1 (with up to 0.276 substitutions/site) to be present simultaneously. These findings support recent observations that rabbit numbers are rising again on the Australian continent, leading once more to overlapping rabbit population ranges and allowing a certain degree of migration between rabbit populations, predominantly by young adults (32).

In sum, it appears that the spatial and temporal patterns of RCV-A1 evolution reflect population dynamics in the rabbit host population and, as such, could be used to gather information about contemporary and historical host-to-host contact and migration patterns at a much finer scale than studying the genetic makeup of the rabbit, which despite its very small founder population has retained its genetic diversity (43). Such studies are urgently needed in Australia to develop improved strategies to manage wild rabbit populations and to monitor control efforts. More detailed studies of the molecular epidemiology of RCV-A1 could also help to identify critical areas where increasing rabbit numbers are leading to overlaps of previously isolated populations, highlighting locations where control efforts need to be increased.

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