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Estimating the age of the subfamily *Orthocoronavirinae* using host divergence times as calibration ages at two internal nodes

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ABSTRACT

Viruses of the subfamily *Orthocoronavirinae* can cause mild to severe disease in people, including COVID-19, MERS and SARS. Their most common natural hosts are bat and bird species, which are mostly split across four virus genera. Molecular clock analyses of orthocoronaviruses suggested the most recent common ancestor of these viruses might have emerged either around 10,000 years ago or, using models accounting for selection, many millions of years. Here, we reassess the evolutionary history of these viruses. We present time-aware phylogenetic analyses of a RNA-dependent RNA polymerase locus from 123 orthocoronaviruses isolated from birds and bats, including those in New Zealand, which were geographically isolated from other bats around 35 million years ago. We used this age, as well as the age of the avian-mammals split, to calibrate the molecular clocks, under the assumption that these ages are applicable to the analyzed viruses. We found that the time to the most recent ancestor common for all orthocoronaviruses is likely 150 or more million years, supporting clock analyses that account for selection.

1. Introduction

Orthocoronaviruses (family *Coronaviridae*, subfamily *Orthocoronavirinae*) are infectious agents of birds and mammals. In humans, they can cause mild illness and commonly cause colds, but emergent viruses can cause more severe disease, with Severe Acute Respiratory Syndrome (SARS) (Peiris et al., 2003), Middle Eastern Respiratory Syndrome (MERS) (Zaki et al., 2012) and Coronavirus disease 2019 (COVID-19) (Huang et al., 2020; Wang et al., 2020; Wu et al., 2020) all causing more severe disease and death in varying proportions of cases. The *Coronaviridae* were recently reclassified, specifically the subfamily *Coronavirinae* was renamed to *Orthocoronavirinae*, with many species formally recognized (Gorbalenya et al., 2020). Orthocoronaviruses are positive-sense RNA viruses and are classified into *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* genera (Gorbalenya et al., 2020; de Groot RJ et al., 2011). Alphacoronaviruses and betacoronaviruses are only found in mammals, whereas gammacoronaviruses and deltacoronaviruses mainly infect birds. SARS (Peiris et al., 2003), in particular, initiated the discovery of novel orthocoronaviruses in humans, domesticated animals, and wildlife (Poon et al., 2005; Snijder et al., 2003; Wevers and van der Hoek, 2009; Zaki et al., 2012). Bats and birds host the greatest diversity of these viruses and are the likely natural 'ancestral' reservoirs of the viruses (Wertheim et al., 2013; Wong et al., 2019; Zhou et al., 2021). Previous studies have identified both evidence for possible orthocoronavirus – host codivergence and coevolution as well as recent cross-species transmission events (Leopardi et al., 2018; Zhang et al., 2020).

Molecular clock analyses of the RNA-dependent RNA polymerase (RdRp) gene and five other genomic regions using different models suggest a time of most recent common ancestor (tMRCA) for the orthocoronaviruses of either around 10,000 years ago (Woo et al., 2012) or 293 (95% CI, 190 to 489) million years ago (Wertheim et al., 2013). The large difference between these approaches was due to the first model using viral isolation (tip) dates and substitution models including the general time-reversible substitution model with a four-bin gamma rate distribution (GTR + Γ4), possibly in the absence of a temporal signal (Rieux and Balloux, 2016), and a second accounting for purifying selection. This was achieved through the development of models in HyPhy that model the substitutions using GTR + Γ4 and a branch site random effects likelihood (BS- REL) model (Pond et al., 2011) to account for variation in selection pressure across codon sites and phylogenetic lineages (Wertheim et al., 2013). These models reduce the effect of purifying selection that prevents the estimation of ages (Wertheim and Pond, 2011) through maintaining evidence of sequence homology after

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saturation at synonymous sites. For eukaryotic organisms, recent advances in phylogenetic analyses have allowed the use of fossils to calibrate these clocks (Gavryushkina et al., 2017; Heath, 2012), using fossil dates to calibrate nodes in the phylogenetic tree. Viruses are not fossilized and so tip calibration is usually used. However, endogenous viral elements and host species divergence ages have been used to estimate the age of other viruses, including single stranded, non-retroviral RNA viruses (Supplementary Table S1) (Belyi et al., 2010; Gifford et al., 2008; Gilbert and Feschotte, 2010; Han and Worobey, 2012; McGeoch and Cook, 1994; McGeoch et al., 1995; Suh et al., 2013, 2014; Taylor et al., 2010; Thézé et al., 2011). For example, the divergence of mammal hosts around 39–52 million years ago years ago with related endogenous filovirus elements lead to the estimation that filoviruses may be tens of millions years old, rather than the 10,000 years estimated by tip dates (Taylor et al., 2010).

Here we take advantage of biogeographic theory and mammalian speciation, including the unique features of phylogeography in New Zealand, to calibrate modeling approaches to estimate the age of bat and then representative bat and bird orthocoronaviruses. Specially, *Alphacoronavirus* RdRp RNA has been discovered in *Mystacina tuberculata* bats in New Zealand (Hall et al., 2014; Wang et al., 2015). New Zealand is estimated to have separated from other landmasses some 84 million years ago (Mortimer et al., 2017) and bats were, until the arrival of humans just 700 years ago between 1320 and 1350 (Walter et al., 2017), the only non-marine mammal present on the continent for nearly 35 million years. We assume there is an ancient, coevolutionary relationship between orthocoronaviruses and their bat or bird hosts for this analysis (Gorbalenya, 2008; Gorbalenya et al., 2006; Wertheim et al., 2013).

2. Materials and methods

2.1. Sequence data sets

Orthocoronavirus genomes (n = 123; Table 1) from all four genera were downloaded from GenBank in March 2020. Because only fragments of the RdRp gene (561 bp) were available from the New Zealand bats, we limited our analyses to this genomic region. This has additional benefits because this region is apparently free of recombinant sequences (Wertheim et al., 2013) and is relatively conserved (Ziebuhr et al., 2001). However, we tested nucleotide sequences for evidence of recombination using DualBrothers (Minin et al., 2005). A total of 105 sequences from bat hosts were used for the initial analyses, and a further 18 sequences from bird hosts were included in subsequent tests (Table 1). All nucleotide sequences were aligned at the amino acid level using MAFFT version 7 employing the E-INS-i algorithm (Katoh and Standley, 2013).

2.2. Phylogenetic analyses

We chose to use amino acid sequences in our primary analyses but, due to the short length of the available sequences, we also re-ran analyses using the nucleotides. We used BEAST v1.10.4 (Suchard et al., 2018) and BEAST2 v2.6.3 (Bouckaert et al., 2019) to analyse the amino acid and nucleotide sequence alignments respectively. We did not use tip dates as the timescales mean all isolates are effectively contemporaneously sampled. We initially assumed a constant population size and a strict clock with an LG substitution model for amino acid sequences (only available in BEAST v1) and HKY substitution model as well as a four-bin gamma rate distribution (GTR + Γ4) with a proportion of invariant sites for nucleotides. After some initial tree topology checking to confirm that genera and the New Zealand and Australian bat-derived orthocoronaviruses were monophyletic, we put a calibration prior on the node that is the common ancestor of the New Zealand and Australian bat-derived orthocoronaviruses. To check the sensitivity of our assumptions we then also put a prior on the age of all the bird-derived

Table 1
Orthocoronavirus sequences analyzed in this study.

Genus and published virus name[a]	Host species	Accession	Year	Sampling country
Alphacoronavirus				
Miniopterus bat coronavirus 1	*Miniopterus* spp.	AY864196	2004	Hong Kong
Bat coronavirus HKU7	*Miniopterus magnater*	DQ249226	2005	Hong Kong
Scotophilus bat coronavirus 512	*Scotophilus kuhlii*	DQ648858	2005	China
Bat coronavirus HKU2	*Rhinolophus sinicus*	EF203064	2006	China
Bat coronavirus 1B	*Miniopterus pusillus*	EU420137	2006	China
Bat coronavirus 1A	*Miniopterus magnater*	EU420138	2005	China
Bat coronavirus HKU8	*Miniopterus pusillus*	EU420139	2005	China
Bat coronavirus	*Miniopterus schreibersii*	GU190243	2008	Bulgaria
Bat coronavirus	*Miniopterus schreibersii*	GU190244	2008	Bulgaria
Miniopterus bat coronavirus/Kenya/KY33/2006	*Miniopterus inflatus*	HQ728485	2006	Kenya
Coronavirus BtCoV/KP256/Art_jam/PAN/2010	*Artibeus jamaicensis*	JQ731784	2010	Panama
Coronavirus BtCoV/KP565/Art_jam/PAN/2010	*Artibeus jamaicensis*	JQ731786	2010	Panama
Bat coronavirus	*Miniopterus schreibersii*	KF294269	2012	China
Bat coronavirus	*Miniopterus schreibersii*	KF294270	2012	China
Bat coronavirus	*Miniopterus schreibersii*	KF294271	2012	China
Bat coronavirus	*Miniopterus schreibersii*	KF294275	2012	China
Mystacina coronavirus New Zealand/2013	*Mystacina tuberculata*	KF515987	2013	New Zealand
Mystacina coronavirus New Zealand/2013	*Mystacina tuberculata*	KF515988	2013	New Zealand
Mystacina coronavirus New Zealand/2013	*Mystacina tuberculata*	KF515989	2013	New Zealand
Mystacina coronavirus New Zealand/2013	*Mystacina tuberculata*	KF515990	2013	New Zealand
Alphacoronavirus BtCoV/MSTM2/Min_nat/RSA/2010	*Miniopterus* cf. *natalensis*	KF843851	2010	South Africa
Alphacoronavirus BtCoV/VH_NC2/Neo_cap/RSA/2012	*Neoromicia* cf. *capensis*	KF843854	2010	South Africa
Alphacoronavirus BtCoV/GrNC1/Neo_cap/RSA/2012	*Neoromicia* cf. *capensis*	KF843855	2010	South Africa
Alphacoronavirus BtCoV/GrNC2/Neo_cap/RSA/2012	*Neoromicia* cf. *capensis*	KF843856	2010	South Africa
BtRf-AlphaCoV/HuB2013	*Rhinolophus ferrumequinum*	KJ473807	2013	China
BtMs-AlphaCoV/GS2013	*Myotis* sp.	KJ473810	2013	China
229E-related bat coronavirus	*Hipposideros abae*	KT253270	2010	Ghana
229E-related bat coronavirus	*Hipposideros abae*	KT253272	2010	Ghana
229E-related bat coronavirus	*Hipposideros* cf. *ruber*	KT253273	2010	Ghana
229E-related bat coronavirus	*Hipposideros abae*	KT253274	2010	Ghana
229E-related bat coronavirus	*Hipposideros* cf. *ruber*	KT253297	2011	Ghana
229E-related bat coronavirus	*Hipposideros* cf. *ruber*	KT253298	2011	Ghana
		KU343194	2012	China

(*continued on next page*)

Table 1 (*continued*)

Genus and published virus name[a]	Host species	Accession	Year	Sampling country
Bat coronavirus MsBtCoV/4039	*Miniopterus schreibersii*			
Bat coronavirus HpBtCoV/3723	*Hipposideros pomona*	KU343196	2012	China
Bat coronavirus RaBtCoV/4307-1	*Rhinolophus affinis*	KU343197	2013	China
Bat coronavirus	*Myotis siligorensis*	KY770850	2013	China
Bat coronavirus	*Myotis davidii*	KY770851	2013	China
Bat coronavirus	*Hipposideros armiger*	KY770852	2013	China
Bat coronavirus	*Rhinolophus pearsonii*	KY770853	2013	China
Bat coronavirus	*Rhinolophus macrotis*	KY770854	2013	China
Bat coronavirus	*Myotis davidii*	KY770856	2013	China
Bat coronavirus	*Ia io*	KY770857	2013	China
Alphacoronavirus sp.	*Neoromicia capensis*	MF593271	2013	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG193605	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG193610	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG193611	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG193616	2016	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG205585	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG205586	2014	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG205590	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG205592	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG205598	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG205599	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG252860	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG252863	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG252866	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG252870	2016	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG252871	2016	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG252874	2016	South Africa
Bat alphacoronavirus	*Neoromicia nanus*	MG252875	2016	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG310222	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG310232	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG310235	2015	South Africa
Bat alphacoronavirus	*Miniopterus natalensis*	MG310236	2016	South Africa
Bat alphacoronavirus	*Miniopterus natalensis*	MG310239	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG310241	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG310242	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG310246	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG310247	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG310253	2014	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG310257	2014	South Africa

Table 1 (*continued*)

Genus and published virus name[a]	Host species	Accession	Year	Sampling country
Bat alphacoronavirus	*Neoromicia capensis*	MG817491	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG817498	2015	South Africa
Bat alphacoronavirus	*Neoromicia capensis*	MG817499	2015	South Africa
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687934	2014	Vietnam
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687937	2014	Vietnam
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687938	2014	Vietnam
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687941	2014	Vietnam
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687942	2014	Vietnam
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687943	2014	Vietnam
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687944	2014	Vietnam
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687945	2014	Vietnam
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687946	2014	Vietnam
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687948	2014	Vietnam
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687954	2014	Vietnam
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687955	2014	Vietnam
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687956	2014	Vietnam
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687957	2014	Vietnam
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687958	2014	Vietnam
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687960	2014	Vietnam
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687961	2014	Vietnam
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687965	2015	Vietnam
Alphacoronavirus sp.	*Scotophilus kuhlii*	MH687966	2015	Vietnam
Bat coronavirus	*Pipistrellus pipistrellus*	MH921428	2016	China
Bat coronavirus	*Pipistrellus pipistrellus*	MH921429	2016	China
Coronavirus BtSk-AlphaCoV/GX2018B	*Scotophilus kuhlii*	MK211370	2017	China
Coronavirus BtSk-AlphaCoV/GX2018D	*Scotophilus kuhlii*	MK211372	2017	China
Chalinolobus morio alphacoronavirus	*Chalinolobus morio*	MN602059	2016	Australia
Chalinolobus morio alphacoronavirus	*Chalinolobus morio*	MN602060	2016	Australia
Miniopterus pusillus bat coronavirus	*Miniopterus pusillus*	MN611518	2018	China
Scotophilus kuhlii bat coronavirus 512-related	*Scotophilus kuhlii*	MN611521	2018	China
Betacoronavirus				
Bat SARS coronavirus[c]	*Rhinolophus pearsoni*	DQ071615	2004	China
Bat coronavirus/133/2005	*Tylonycteris pachypus*	DQ648794	2005	China
Bat coronavirus HKU5	*Pipistrellus abramus*	EF065512	2006	China
Bat coronavirus HKU9	*Rousettus leschenaulti*	HM211100	2006	China
Gammacoronavirus				
Infectious bronchitis virus	*Gallus gallus*	FJ904719	1991	USA
Infectious bronchitis virus	*Gallus gallus*	FJ904721	1972	USA
Turkey coronavirus	*Meleagris gallopavo*	GQ427173	2003	USA
Turkey coronavirus	*Meleagris gallopavo*	GQ427175	1994	USA
Turkey coronavirus	*Meleagris gallopavo*	GQ427176	1998	USA
Infectious bronchitis virus	*Gallus gallus*	GQ504724	1941	USA
Infectious bronchitis virus	*Gallus gallus*	GU393336	1954	USA
Duck coronavirus	*Duck*[f]	JF705860	2004	China
Infectious bronchitis virus	*Gallus gallus*	JF828980	2010	China
Deltacoronavirus				
Bulbul coronavirus HKU11	*Pycnonotus jocosus*	FJ376619	2007	China
		FJ376621	2007	China

(*continued on next page*)

Table 1 (*continued*)

Genus and published virus name[a]	Host species	Accession	Year	Sampling country
Thrush coronavirus HKU12	*Turdus hortulorum*			
Munia coronavirus HKU13	*Lonshura striata*	FJ376622	2007	China
White-eye coronavirus HKU16	*Zosterops* sp.	JQ065044	2007	China
Sparrow coronavirus HKU17	*Passer montanus*	JQ065045	2007	China
Magpie robin coronavirus HKU18	*Copsychus saularis*	JQ065046	2007	China
Night-heron coronavirus HKU19	*Nycticorax nycticorax*	JQ065047	2007	China
Wigeon coronavirus HKU20	*Anas Penelope*	JQ065048	2008	China
Common-moorhen coronavirus HKU21	*Gallinula chloropus*	JQ065049	2007	China

[a] Species, strain/isolate names are in Supplemental Table S2.

viruses and subsequently only the bird derived orthocoronaviruses, so in total used two calibration points in three scenarios: a bat-only, a bird-bat, and a bird-only. The bat-only calibration was tested on two datasets, one complete (123 sequences from bat and bird hosts) and one using only bat hosts (105 sequences). The ages we used were 35 million years (2.5–97.5% quartiles = 30–42 MY) for the New Zealand bats, based on the estimated divergence time of *Mysticina* from other bats (Van den Bussche and Hoofer, 2000), and 150 million years (2.5–97.5% quartiles = 134–167 MY) for the age of birds (Hu et al., 2009). However, because New Zealand has one other living bat species, *Chalinolobus tuberculatus*, we also test the sensitivity of this divergence time, and use the age of *C. tuberculatus*, 17 million years (2.5–97.5% quartiles = 14–20 MY) (Dool et al., 2016). We identify calibrations with the taxa (e.g. bird, bat) and the age in superscript^{x}, e.g. bat^{35} is the 35MY calibration on the New Zealand-Australian bat virus ancestor node. Lastly, we also used a relaxed clock for each calibration scenario and general time-reversible substitution model with a four-bin gamma rate distribution (GTR + Γ4) for each nucleotide scenario. Strict clock models were run for 10 million MCMC samples with a 10% burnin and sampling every 1000, whereas the relaxed clocks require longer MCMC chains, so were run for 100 million and sampled every 10000. The analyses covered 186 amino acid and 561 nucleotide sites. All xml files are available at https://github.com/dtsh2/coronavirus_ancestry and can be replicated. Logs were visualized in Tracer 1.7.1 (Rambaut et al., 2018) and trees plotted in FigTree 1.4.4 (Rambaut, 2012). We checked for overall host-virus coevolution using parafit (Legendre et al., 2002) in the ape R package (Paradis and Schliep, 2019) using taxa (or sister taxa) from the TimeTree database (Hedges et al., 2015) and amino acid model with the bat^{35} and bird^{150} calibrations (see Supplementary Fig. S1). Further manipulation and visualization were performed in R v4.0.4 using beastie (du Plessis, 2020); ggplot2 (Wickham, 2016); ggmcmc (Fernandez-i-Marin, 2016); stringr (Wickham, 2019); and ggdistribute (Burling, 2018) packages.

3. Results

3.1. Assumptions and model adequacy

We found no support for recombination across the RdRp fragment used (see Supplementary Fig. S2). As anticipated, the limited length of amino acid sequences did not provide information for the modelling of some parameter and as a result several of the relaxed clock models returned effective samples sizes (ESS) of <200. However, crucially the ESS for tree heights (ages) was >1179 (range 1179–5036). Using nucleotide sequences, all chains converged with ESS all >200, with all ESS for tree heights (ages) > 479 (range 479–5775). Overall, the nucleotide tree topology was well supported, with high posterior

TABLE 2
Orthocoronavirus ages by different models in this study using amino acid sequences. Calibration confidence intervals at 2.5–97.5% are 14–20 MY for Bat-17 MYA, 30–42 MY for Bat-35 MYA and 134–167 MY for Bird-150 MYA. Mean and 95% highest posterior density (HPD) only are shown for the substitution rate (per site per million years).

Orthocoronaviruses																
Hosts	Bat	Bat & Bird	Bat & Bird	Bat	Bat & Bird	Bat & Bird	Bat	Bat & Bird	Bat & Bird	Bat	Bat & Bird	Bat & Bird	Bat	Bat & Bird	Bat & Bird	Bat & Bird
Model																
Clock	Relaxed[a]	Relaxed	Relaxed	Relaxed[a]	Relaxed[a]	Relaxed	Strict	Strict	Strict	Strict	Strict	Strict	Strict	Strict	Strict	Strict
Site model	LG	LG	LG	LG	LG	LG	LG	LG	LG	LG	LG	LG	LG	LG	LG	LG
Calibration	Bat-17 MYA	Bat-17 MYA	Bat-17 MYA + Bird-150 MYA	Bat-35 MYA	Bat-35 MYA	Bat-35 MYA + Bird-150 MYA	Bird-150 MYA	Bat-17 MYA	Bat-17 MYA	Bat-17 MYA + Bird-150 MYA	Bat-35 MYA	Bat-35 MYA	Bat-35 MYA	Bat-35 MYA + Bird-150 MYA	Bird-150 MYA	Bird-150 MYA
Age																
Mean	178.5	239.2	176.9	347.6	492	234.7	168	213.7	264.9	167.8	417.8	531.7	185.9	163.4		
Median	160.4	223.4	170	305.8	465.2	218.4	162.8	198	246	166.4	391.3	493.2	184.4	162		
95% HPD	21.2–375.9	85.6–423.9	136–231	38.2–748.1	221.5–832.4	141.5–361.7	134.4–210	90.7–363.4	123.8–446.7	143.1–197.2	190.9–708	240.7–899.1	153.9–219.8	139–191.5		
Range	14.9–1565.5	34.4–1077.3	126–765	31.2–3176.9	92.2–1504.1	136.2–792.2	129.1–454.8	68.5–942.8	72.2–760.4	121.5–240.3	106.8–1265.2	195.5–2897.1	139.1–262.8	124.8–240		
Bat Clade Age																
Mean	178.5	171.3	139.7	347.6	358.9	171.1	131	213.7	199.8	139.7	417.8	399.3	160	134.7		
Median	160.4	160.3	137	305.8	340.6	165.5	129.4	198	186.4	138.4	391.3	367.2	158.9	133.2		
95% HPD	21.2–375.9	61.9–306.1	97–187	38.2–748.1	164.5–603.1	93.4–259.8	89.8–173.4	90.7–363.4	92.3–338.1	108.1–172.6	190.9–708	185.5–687.6	123.9–198	105.5–167.8		
Range	14.9–1565.5	23.4–711.8	68–352	31.2–3176.9	63.6–1127.4	54.6–560.5	67.6–350.4	68.5–942.8	85.8–829.2	88.5–215.2	106.8–1265.2	134.9–2095	101.2–236.5	88.1–218.5		
Substitution rate																
Mean	2.41E-3	1.81E-3	2.20E-3	1.18E-3	9.02E-4	1.81E-3	2.36E-3	1.52E-3	1.63E-3	2.20E-3	7.71E-4	8.13E-4	1.91E-3	2.30E-3		
95% HPD	5.84E-4	8.29E-4	1.65E-3	2.50E-4	3.71E-4	1.04E-3 –2.69E-	1.67E-3	6.79E-4	7.17E-4	1.79E-3 -2.60E-	3.41E-4	3.63E-4	1.55E-3 -2.27E-	1.86E-3		
	–5.49E-3	–2.94E-3	–2.75E-3	–2.79E-3	–1.46E-3	3	-2.99E-3	-2.51E-3	-2.62E-3	3	-1.27E-3	-1.32E-3	3	-2.75E-3		

[a] Low ESS (<200) for some parameters, see text.

support for many nodes. There was support for coevolution between viruses and hosts over the whole tree using parafit (p value = 0.03, Supplementary Fig. S3). The posterior distributions of the HKY and GTR were essentially the same and, as expected there is increasing uncertainty of tMRCA estimates and lower node support with deeper nodes (see Supplementary Table S3, Supplementary Figs. S4–S33). Herein only the amino acid sequences using the 35 MY bat prior (bat^{35}) are discussed, but all the results can be seen in Table 2 and the supplementary information.

3.2. Time to the most recent common ancestor

The strict clock analyses using the bat^{17}, bat^{35}, bat^{17} and $bird^{150}$, bat^{35} and $bird^{150}$ or $bird^{150}$ only calibration points and LG model, estimated bat orthocoronaviruses to be somewhere from 133 to 391 million years old (median values). These values overlapped with the estimates from all other models for the relaxed clocks (Fig. 1). The relaxed clock models had great uncertainty, for example for the bat^{35} only calibration with both bat and bird viruses the estimate was 305MY (38–748 95% HPD). The strict clock estimates were 391MY (190–708 95% HPD). All other values are in Table 2. Overall, our calibrations led to the substitution rates ranging from means of 2.4 $*10^{-3}$ to 7.7 $*10^{-4}$ per MY (see Table 2, Supplementary Table S4 and Fig. S34).

For all analyses, the single younger bat calibration point (17 MY) led to less uncertainty and younger tMRCA estimates if used alone. For the strict clock analyses the differences between estimates when the bird calibration point was used led to non-overlapping 95% HPD. However, in all analyses the estimates for the tMRCA for bat orthocoronaviruses is older than bats themselves (around 50MY, Fig. 2) and always includes the estimated age of birds (150MY), the only exception being the most uncertain strict clock analysis mentioned above with only the 35MY NZ-Australian bat clade prior (bat^{35}), that estimates the viruses to be older. In all cases the estimated orthocoronavirus crown tMRCA is similar to those of bat orthocoronaviruses. The calibration points force the nodes to be monophyletic and in most analyses the maximum clade credibility (MCC) trees had bat (alpha- and betacoronaviruses) with one common ancestor and bird (gamma- and deltacoronaviruses) with another. However, with only the bat calibration point, both the relaxed and strict clocks placed the bird virus ancestors as ancestral to bat viruses, with the switch only once occurring when both calibration points were used using the relaxed clock and GTR+Γ4 model. All 14 amino acid and 16 nucleotide trees with the 35 million year old prior and their node support, 95% HPD and virus names are provided in the supplementary information.

4. Discussion

Our results support previous findings that orthocoronaviruses evolved millions of years ago (Wertheim et al., 2013). Increasingly analyses suggest that many viruses are ancient. Amniotes (reptiles, birds, and mammals) are estimated from fossil and molecular evidence to be around 325 million years old (Blair and Hedges, 2005; Shedlock and Edwards, 2009), whereas birds share an ancestor around 150 million years and bats around 50 million years ago (Simmons et al., 2008;

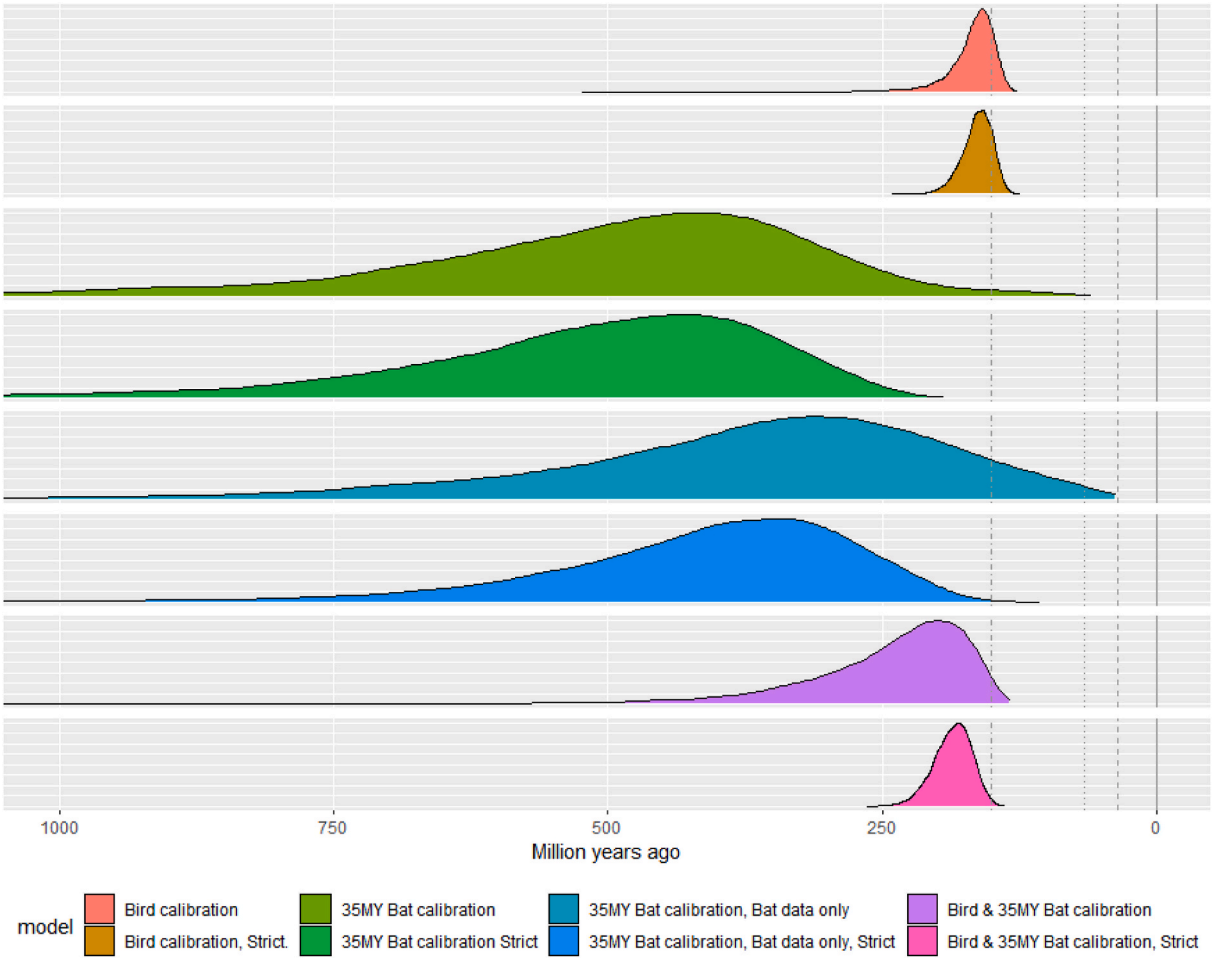

Fig. 1. Crown date estimates for all amino acid models. A 35 MY bat calibration prior on the New Zealand and Australian bat derived virus clade was used. The present (solid), 35MY calibration (dashed), 50MY (approximate age of bats, dotted) and 150MY (approximate age of bats, dash-dot) are shown by vertical bars.

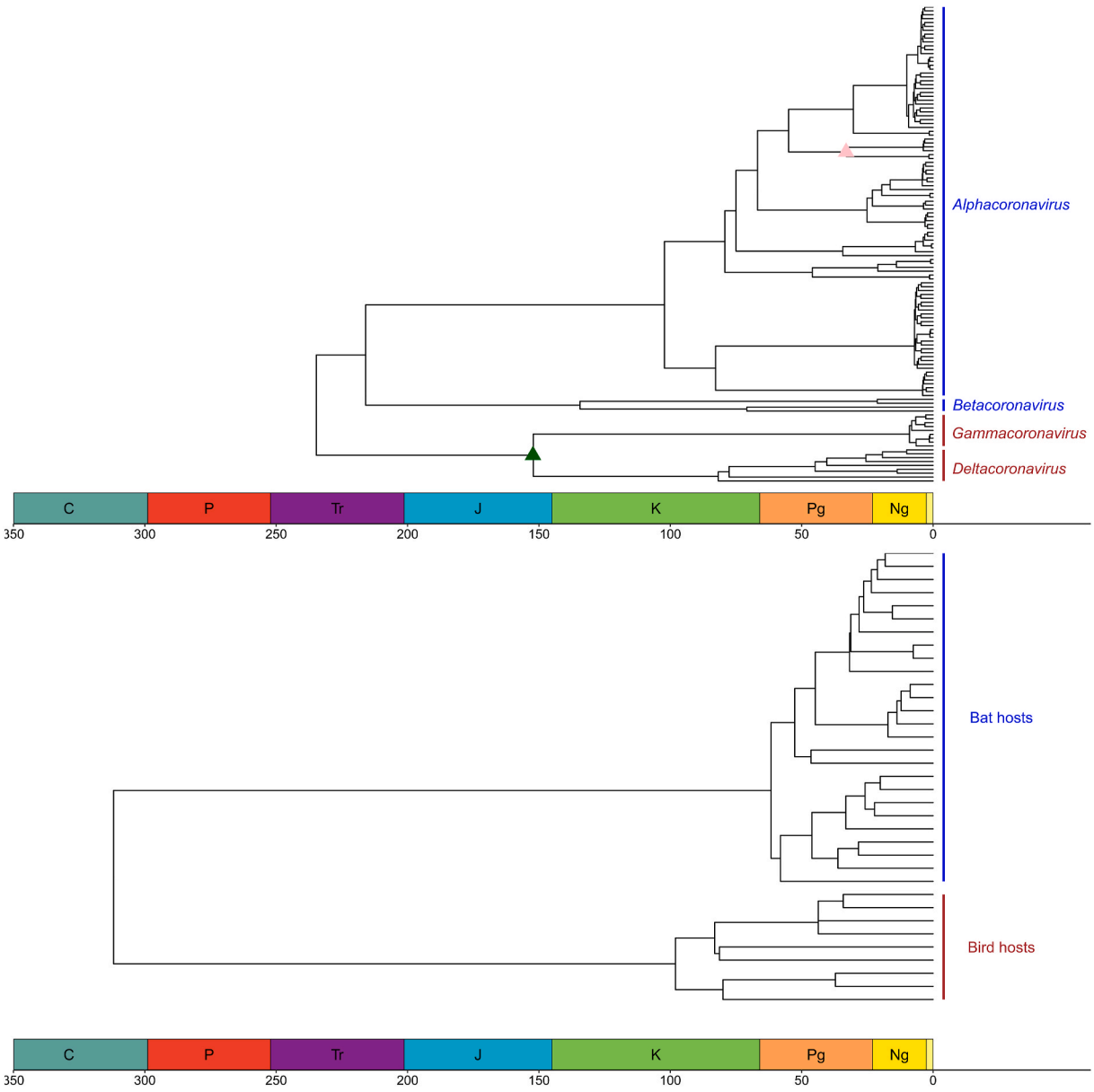

Fig. 2. Phylogeny of orthocoronaviruses and their hosts. Amino acid alignments with a relaxed molecular clock, 35MY bat calibration prior (pink triangle) on the New Zealand and Australian bat derived virus clade and 150MY bird calibration prior on delta- and gammacoronaviruses were used (dark green triangle). Host ages are provided for comparison from http://www.timetree.org/(Hedges et al., 2015) (see Supplementary Fig. S1). Bat viruses and bat hosts (blue) and bird viruses and bird hosts (brown) names are coloured.

Teeling et al., 2005). Our estimated tMRCA dates for orthocoronaviruses of somewhere from 133 to 391 million years ago (median estimates), older than bats and (mostly) birds ancestors, suggesting these viruses evolved prior to bat and possibly bird ancestors among earlier Amniotes. There is, of course, great uncertainty in our estimates (Table 2, Fig. 1), but our results were robust to changes in the use of calibration point position. While the values and uncertainty changed, the use of calibrations either closer to tree tips (bat[17], bat[35]) or deeper in the tree (bird[150]) nearer the crown of all orthocoronaviruses all led to ages older than bats and, mostly, birds. The family *Coronaviridae* includes *Letovirinae* subfamily viruses from a frog (Bukhari et al., 2018) and metatranscriptomic sequencing has identified distinct and diverse *Coronaviridae* phylogenetic groups among jawless and bony fish, providing further evidence that these viruses have evolved with vertebrate hosts over millions of years (Miller et al., 2021; Mordecai et al., 2019).

Here we also assumed these viruses were exclusively bat and bird viruses. It is feasible that other hosts (for example porcine, rodent, etc.) will come to light since host switching does occur, as evidenced by the recent emergence of orthocoronaviruses causing SARS, MERS and COVID-19 in people and swine acute diarrhoea syndrome in pigs (Zhou et al., 2018), and the widespread distribution of other now endemic orthocoronaviruses, such as HCoV-229E. We excluded other viruses because of this, but our results may have influenced by such host switches among bat taxa. The monophyletic relationships of orthocoronaviruses in bats (i.e., alphacoronaviruses and betacoronaviruses) and birds (gammacoronaviruses and deltacoronaviruses), however, suggest these relationships are old and real (Chu et al., 2011; Wong et al., 2019; Woo et al., 2012). The sequencing of additional and/or more complete genomes of bat-derived orthocoronaviruses from islands, especially in the Pacific, may help support these findings.

It is most likely orthocoronaviruses arrived in New Zealand with the colonizing *Mysticina* (~35MYA) or *Chalinolobus* (~17MYA) bats, though the arrival through other non-bat species such as marine mammals or with humans is possible. Given the isolated population of *Mysticina* on a

New Zealand South Island island in the Pacific Ocean the alphacoronavirus RdRp RNA was isolated from and the widespread distribution of bat orthocoronaviruses globally, this later arrival seems unlikely. Further, more recent host switching from non-bat (e.g. rodent) hosts is possible but highly unlikely given the close association of New Zealand bat orthocoronaviruses with Australian bat alphacoronaviruses and the very recent arrival of the first rodents to New Zealand. Rodents (specifically the Polynesian rat (*Rattus exulans*), known to Māori as kiore) only arrived in New Zealand from Polynesia with Māori, not via Australia, approximately 700 years ago, and other rodents only with Europeans. Therefore, it is more likely our results support those using the BS-REL tree calibration and that orthocoronaviruses have been infecting bats and birds for millions of years and, possibly, since their Amniote ancestors diverged approximately 300 million years ago.

There remain other issues to resolve for orthocoronaviruses. For example, while the partial RdRp gene we used was likely free of recent recombination, recombination among orthocoronaviruses is common and can even include genetic material from unrelated viruses, impacting any estimates relating to ancestry (Paskey et al., 2020). By using calibration points so far in the past, we automatically reduce the substitution rate (range $2.4 * 10^{-3}$ to $7.7 * 10^{-4}$per MY). These rates had previously been estimated to be 10^{-5} to 10^{-6} mutations per site per replication (Eckerle et al., 2010) or around 10^{-3} substitutions per site per year (Hon et al., 2008; Wertheim et al., 2013). When clocks become unreliable, therefore, is unknown, though clearly analyses of more recent ancestries like SARS-coronaviruses are most reliable of all (Boni et al., 2020). Time dependent rates of molecular evolution have been observed from a wide range of taxa with short-term substitution rates exceeding long-term by an order of magnitude or more (Ho et al., 2011). Rates of molecular evolution in RNA viruses may span several orders of magnitude (Duffy et al., 2008) with this rapid accumulation of genetic differences in the short-term primarily due to the fidelity of the polymerase that is used during replication, though genome size and replication speed are also important factors. In the longer term, it is thought that strong purifying selection constrains the substitution rate (Duchêne et al., 2014; Holmes, 2003). Together, the evidence supports time varying rates of evolution and so substitution rates (including ours) need to be used and interpreted with caution (Duchêne et al., 2014).

In summary, our analyses using Bayesian evolutionary and tree analyses and mammalian tMRCA estimates have allowed us to make inferences about the age of orthocoronaviruses and support our intuition that orthocoronaviruses probably have an evolutionary history that matches their vertebrate hosts.

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CRediT authorship contribution statement

David T.S. Hayman: Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft, preparation. **Matthew A. Knox:** Data curation, Formal analysis, Writing – review & editing.

Declaration of competing interest

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2021.08.004.

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