

1 **Faecal virome of the Australian grey-headed flying fox from**
2 **urban/suburban environments contains novel coronaviruses,**
3 **retroviruses and sapoviruses**

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26 **ABSTRACT**

27 Bats are important reservoirs for viruses of public health and veterinary concern. Virus
28 studies in Australian bats usually target the families *Paramyxoviridae*, *Coronaviridae* and
29 *Rhabdoviridae*, with little known about their overall virome composition. We used
30 metatranscriptomic sequencing to characterise the faecal virome of grey-headed flying foxes
31 from three colonies in urban/suburban locations from two Australian states. We identified
32 viruses from three mammalian-infecting (*Coronaviridae*, *Caliciviridae*, *Retroviridae*) and
33 one possible mammalian-infecting (*Birnaviridae*) family. Of particular interest were a novel
34 bat betacoronavirus (subgenus *Nobecovirus*) and a novel bat sapovirus (*Caliciviridae*), the
35 first identified in Australian bats, as well as a potentially exogenous retrovirus. The novel
36 betacoronavirus was detected in two sampling locations 1,375 km apart and falls in a viral
37 lineage likely with a long association with bats. This study highlights the utility of unbiased
38 sequencing of faecal samples for identifying novel viruses and revealing broad-scale patterns
39 of virus ecology and evolution.

40

41 **Keywords**

42 Coronavirus, sapovirus, retrovirus, faecal, mammalian, grey-headed flying fox

43 **1. Introduction**

44 Bats (order Chiroptera) are one of the largest mammalian orders with a unique physiology
45 adapted for flight. The number of bat colonies in urban habitats has increased in recent
46 decades, leading to more frequent interactions with humans, companion animals and
47 livestock that have in turn facilitated outbreaks of zoonotic disease (Plowright et al., 2011).
48 This process has been dramatically highlighted by the emergence of severe acute respiratory
49 syndrome coronavirus 2 (SARS-CoV-2) and the detection of SARS-like coronaviruses in
50 Asian bat populations (Temmam et al., 2022, Zhou et al., 2021, Zhou et al., 2020,
51 Wacharapluesadee et al., 2021, Murakami et al., 2020). In addition, bats have been associated
52 with the emergence of Hendra virus (Halpin et al., 2000), Nipah virus (Yob et al., 2001),
53 lyssaviruses (Botvinkin et al., 2003, Gould et al., 1998) and SARS-CoV (Li et al., 2005). In
54 turn, these outbreaks have led to increased sampling of bat species, and the widespread use of
55 metagenomic sequencing has enabled more detailed exploration of the bat virome (Wu et al.,
56 2016, Hardmeier et al., 2021, Van Brussel and Holmes, 2022).

57
58 In Australia, bat species of the genus *Pteropus* are reservoir hosts for Hendra virus and
59 Menangle virus, zoonotic pathogens of the family *Paramyxoviridae* (Halpin et al., 2000,
60 Philbey et al., 1998), as well as Australian bat lyssavirus, a zoonotic virus of the
61 *Rhabdoviridae* that causes rabies in mammals (Gould et al., 1998). Studies of viruses in bats
62 in Australia have largely focused on these virus families and recently identified a new
63 member of the *Paramyxoviridae* – Cedar virus – as well as a novel genotype of Hendra virus
64 (Wang et al., 2021, Marsh et al., 2012). Although important, these studies lack information
65 on overall virome composition, particularly those virus families not included in targeted PCR
66 studies.

67
68 The grey-headed flying fox (*Pteropus poliocephalus*), a member of the megabat family
69 Pteropodidae and native to Australia, is a species of importance in the context of zoonotic
70 viruses. Grey-headed flying foxes are distributed throughout the eastern coastline of Australia
71 (Queensland, New South Wales and Victoria) and more recently a colony was established in
72 Adelaide (South Australia). Grey-headed flying foxes feed on fruit, pollen and nectar and
73 roost in large colonies, sometimes sharing roosting locations with other species of *Pteropus*,
74 allowing intraspecies and interspecies virus transmission (Timmiss et al., 2021). Roosting
75 sites are commonly located alongside human communities including in densely populated

76 urban settings (Williams et al., 2006). As numerous viruses are transmitted by faeces and
77 other excretions, the co-habitation between bats and humans likely increases the risk of
78 zoonotic spill-over.

79

80 Herein, we used metatranscriptomic sequencing of faecal samples to describe the community
81 of viruses present in the gastrointestinal tract of grey-headed flying foxes from three
82 sampling locations in two Australian states – Centennial Park and Gordon in Sydney, New
83 South Wales, and the Botanic Park, Adelaide in South Australia. Specifically, to reveal the
84 composition and abundance of viruses in bats residing in metropolitan areas we sampled
85 roosting sites either located in a residential setting or in parks that are frequented by humans.

86

87 **2. Methods**

88 **Sample collection**

89 Faecal samples were collected from grey-headed flying fox roosting sites in three regions of
90 Australia: Centennial Parklands, Centennial Park New South Wales (NSW), Gordon NSW,
91 and Botanic Park, Adelaide parklands, Adelaide, South Australia (Table 1, Fig. 1A).

92 Sampling was conducted over two dates in 2019 for the Centennial Park and Gordon sites,
93 while the roosting site in the Adelaide parklands was sampled over several months in 2019
94 (Table 1). A plastic sheet of approximately 3 x 5 metres was placed under densely populated
95 trees the night before collection. The following morning samples captured by the plastic sheet
96 were placed into 2 mL tubes and immediately stored at -80°C until processing. Any faecal
97 sample touching or submerged in urine was discarded.

98

99 **Table 1.** Sampling overview, including number of samples allocated to sequencing pools and
100 sequencing metadata.

Location	Sampling date	Pool no.	No. of samples	No. of reads	No. of contigs
Centennial Park, NSW 33. 89999°S, 151.23592°E	5 February 2019	01	12	24,732,494	159,527
		02	9	35,835,953	147,425
		03	9	31,960,624	107,431
	26 February 2019	04	9	19,833,973	111,196
		05	11	31,410,836	136,180
		06	9	29,318,213	105,118

		07	10	19,160,704	90,339
Gordon, NSW 33.75065°S, 151.16242°E	12 March 2019	01	12	52,605,108	89,247
		02	12	48,784,843	50,574
		03	9	27,396,450	118,509
	26 March 2019	04	11	36,591,148	181,524
		05	12	36,815,461	146,466
		06	12	52,934,611	97,013
		07	10	37,980,832	156,960
Adelaide, SA 34.91571°S, 138.6068°E	2019	01	8	25,977,712	135,969
	2019	02	9	21,113,731	113,546

101

102

103 **RNA extraction, sequencing and read processing**

104 Faecal samples were homogenised using the Omni Bead Ruptor 4 with 1.44 mm ceramic
105 beads (Omni international). Total RNA was extracted from each sample individually using
106 the RNeasy Plus Mini Kit (Qiagen) following the manufacturer’s protocol. RNA was pooled
107 in equimolar ratios and separated by sampling location, date and RNA concentration (Table
108 1). Ribosomal RNA was depleted, and libraries constructed using the Illumina Stranded Total
109 RNA Prep with Ribo-Zero Plus (Illumina) preparation kit. Libraries were sequenced as 150
110 bp paired-end on the Illumina Novaseq 6000 platform at the Australian Genome Research
111 Facility (AGRF). Read ends with a quality score of below 25 phred and adapter sequences
112 were removed using cutadapt v1.8.3 (Kechin et al., 2017). Sortmerna v4.3.3 was used to
113 remove 5S and 5.8S, eukaryotic 18S and 23S, bacterial 16S and 23S, and Archaea 16S and
114 23S ribosomal RNA (rRNA) reads (Kopylova et al., 2012). The filtered reads were then *de*
115 *nov*o assembled using Megahit v1.1.3 (Li et al., 2015) and contigs were compared to the non-
116 redundant protein database using diamond v2.0.9. The Genemark heuristic approach
117 (Besemer and Borodovsky, 1999, Zhu et al., 2010) and information from closely related
118 viruses were used to predict genes and annotate genomes. Intact retrovirus genomes were
119 detected using an in-house pipeline (Chang et al., manuscript in preparation). The Geneious
120 assembler (Geneious Prime version 2022.1.1) was used to reassemble megahit contigs from
121 multiple libraries for bat faecal associated retrovirus 2 (see Results). The final sequence for
122 bat faecal associated retrovirus 2 (see Results) was determined by mapping reads from all

123 libraries to the reassembled genome on Geneious Prime using a 0% (majority) threshold for
124 the final consensus sequence.

125

126 **Abundance estimation**

127 Virus and host abundance were estimated by mapping non-rRNA reads from each library to
128 assembled contigs, and to the COX1 gene (accession no. KF726143) from the *P. alecto*
129 (Black flying fox) genome using Bowtie2 v2.3.4.3 (Langmead and Salzberg, 2012). The
130 impact of index-hopping was minimised by excluding the read abundance count for a contig
131 in any library that was less than 0.1% of the highest read count for that assembled contig in
132 any other library.

133

134 **Phylogenetic analysis**

135 Virus amino acid sequences were aligned with related sequences (i.e., representing the same
136 virus family and/or genus) retrieved from the NCBI/GenBank database using MAFFT v7.450
137 (Kato and Standley, 2013) and the E-INS-I algorithm (Kato et al., 2005). The partial RdRp
138 sequence of *P. alecto*/Aus/SEQ/2009 was retrieved from Smith et al. (2016). The gappyout
139 method in TrimAL v1.4.1 was used to remove ambiguous regions in the alignment (Capella-
140 Gutiérrez et al., 2009). Maximum likelihood trees of each data set were inferred using IQ-
141 TREE v1.6.7 (Nguyen et al., 2014), employing the best-fit amino acid substitution model
142 determined by the ModelFinder program (Kalyaanamoorthy et al., 2017) in IQ-TREE. Nodal
143 support was assessed using 1000 ultrafast bootstrap replicates (Hoang et al., 2017). Any virus
144 sequence with over 90% nucleotide similarity to another detected here was excluded from the
145 phylogenetic analysis.

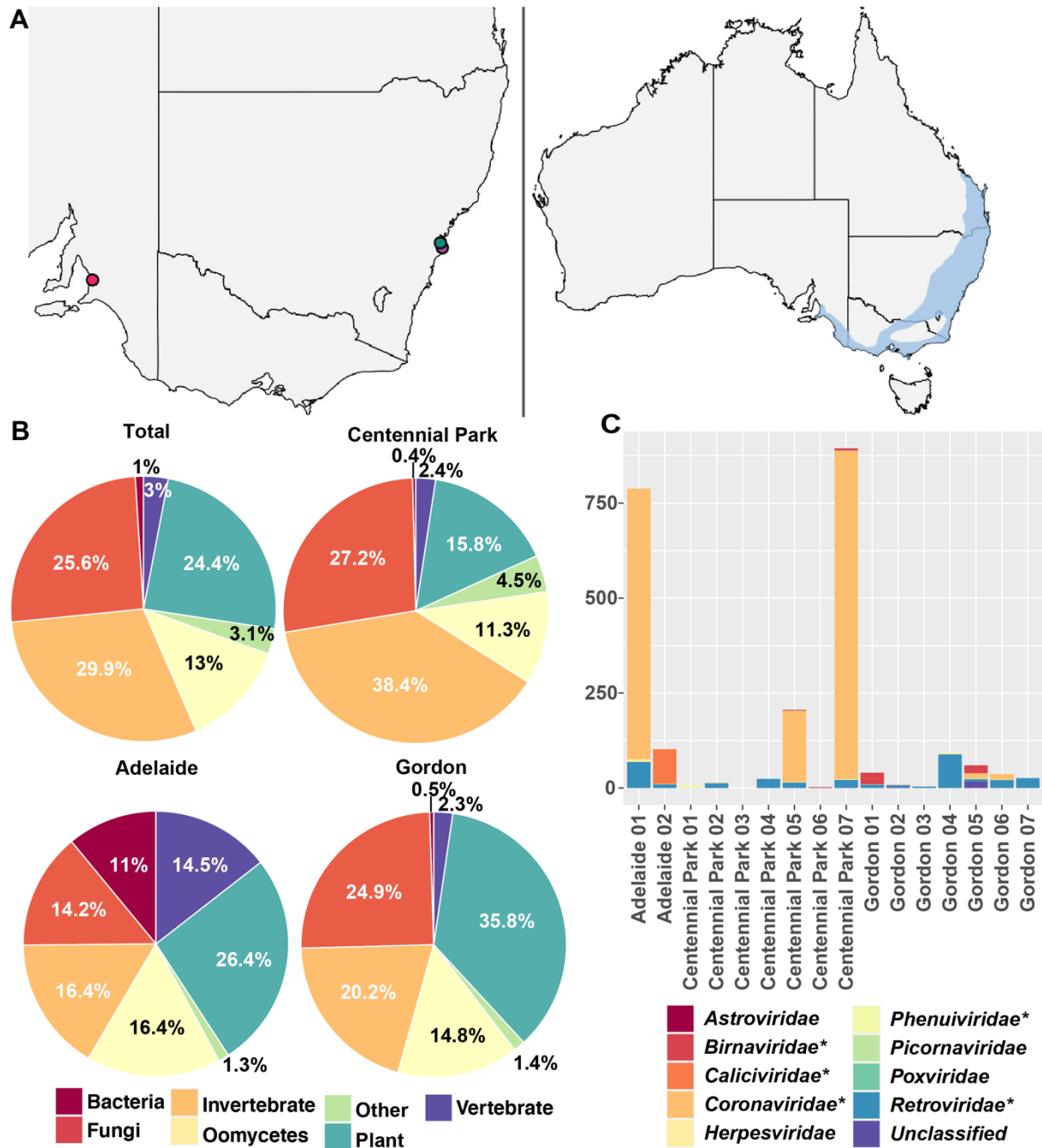
146

147 **3. Results**

148 **Virome overview**

149 In total, 164 faecal samples allocated to 16 libraries underwent metatranscriptomic
150 sequencing. This generated 19,160,704 to 52,934,611 reads per library (average of
151 33,278,293 reads) after read filtering (Table 1). Reads were *de novo* assembled into 50,574 to
152 181,524 contigs (average of 121,689 contigs) per library (Table 1). A total of 5,933 contigs
153 were assigned as of viral origin across all the libraries. The samples collected at Centennial
154 Park, Sydney produced the most viral contigs, with 3,216 identified from 65 virus families
155 (Supplementary Fig. 1). The Gordon, NSW sample site produced 2,399 virus contigs from 66

156 virus families, while the Adelaide site contained 318 virus contigs from 33 virus families,
157 although this site had only two sequencing libraries comprising 17 faecal samples, compared
158 to seven sequencing libraries in each of the other two locations (69 faecal samples from
159 Centennial Park, 78 from Gordon) (Table 1, Supplementary Fig. 1). Screening of the NCBI
160 protein database revealed assembled virus contigs were mostly associated with infection of
161 invertebrates (29.9% of total contigs), fungi (25.6%), plants (24.4%), and oomycetes (13%),
162 representing 79 virus families (Fig. 1B, Supplementary Fig. 1). These viruses were most
163 likely associated with host diet and differed in frequency depending on sampling site (Fig.
164 1B, Supplementary Fig. 1). The plant, fungal, and oomycete-associated viruses, as well as
165 those likely to be bacteriophage (including the picobirnaviruses) were not considered further.
166 Importantly, however, we also identified sequences from viruses likely associated with
167 mammalian infection (3% overall), including near complete genomes from members of the
168 *Coronaviridae*, *Caliciviridae* and *Retroviridae* (Fig. 1B).



169

170

171 **Fig. 1.** Overview of sampling sites and bat faecal sample composition. (A) Sampling
 172 locations in Australia (left) and distribution map of the grey-headed flying fox (right) (IUCN,
 173 2021). (B) Likely hosts of viral contigs based on host designation of the closest relatives in
 174 the NCBI non-redundant protein database. (C) Read abundance presented as reads per million
 175 (RPM) for the vertebrate-associated virus sequences for each library and separated by virus
 176 family. The virus families discussed in this study are highlighted with an asterisk.

177

178 **Mammalian-associated viruses**

179 We detected contigs from nine viral families likely to infect mammals (Fig. 1C). The
180 *Coronaviridae* and *Retroviridae* were particularly abundant and present in five and 13
181 libraries, respectively (Fig. 1C). Members of the *Birnaviridae* and *Caliciviridae* were also
182 abundant in specific libraries (Fig. 1C). The remaining mammalian-associated viral families
183 were only detected at low abundance and the contigs were not of sufficient length for further
184 characterisation.

185

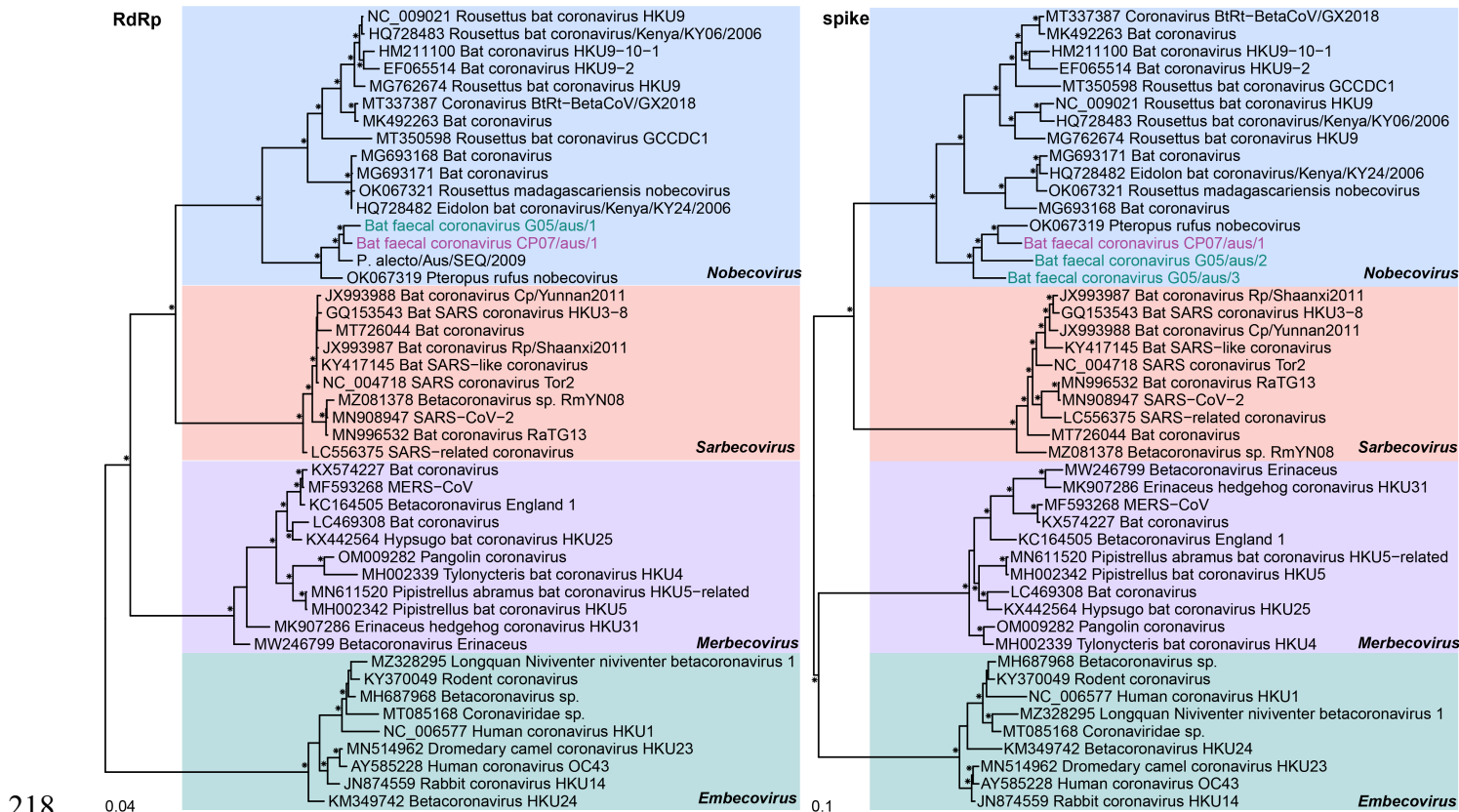
186 **Novel betacoronavirus (*Coronaviridae*)**

187 A novel complete betacoronavirus genome (single-strand, positive-sense RNA virus;
188 +ssRNA) – provisionally denoted bat faecal coronavirus CP07/aus/1 – was identified in a
189 sequencing library sampled from Centennial Park (pool no. 07) and in a sequencing library
190 from Adelaide (pool no. 01). These two sequences exhibited 99.8% identity over the
191 complete viral genome indicating that they represent the same species. Additionally, three
192 sequences with 99.2-100% sequence identity to CP07/aus/1 were identified in an additional
193 Centennial Park library (pool no. 05).

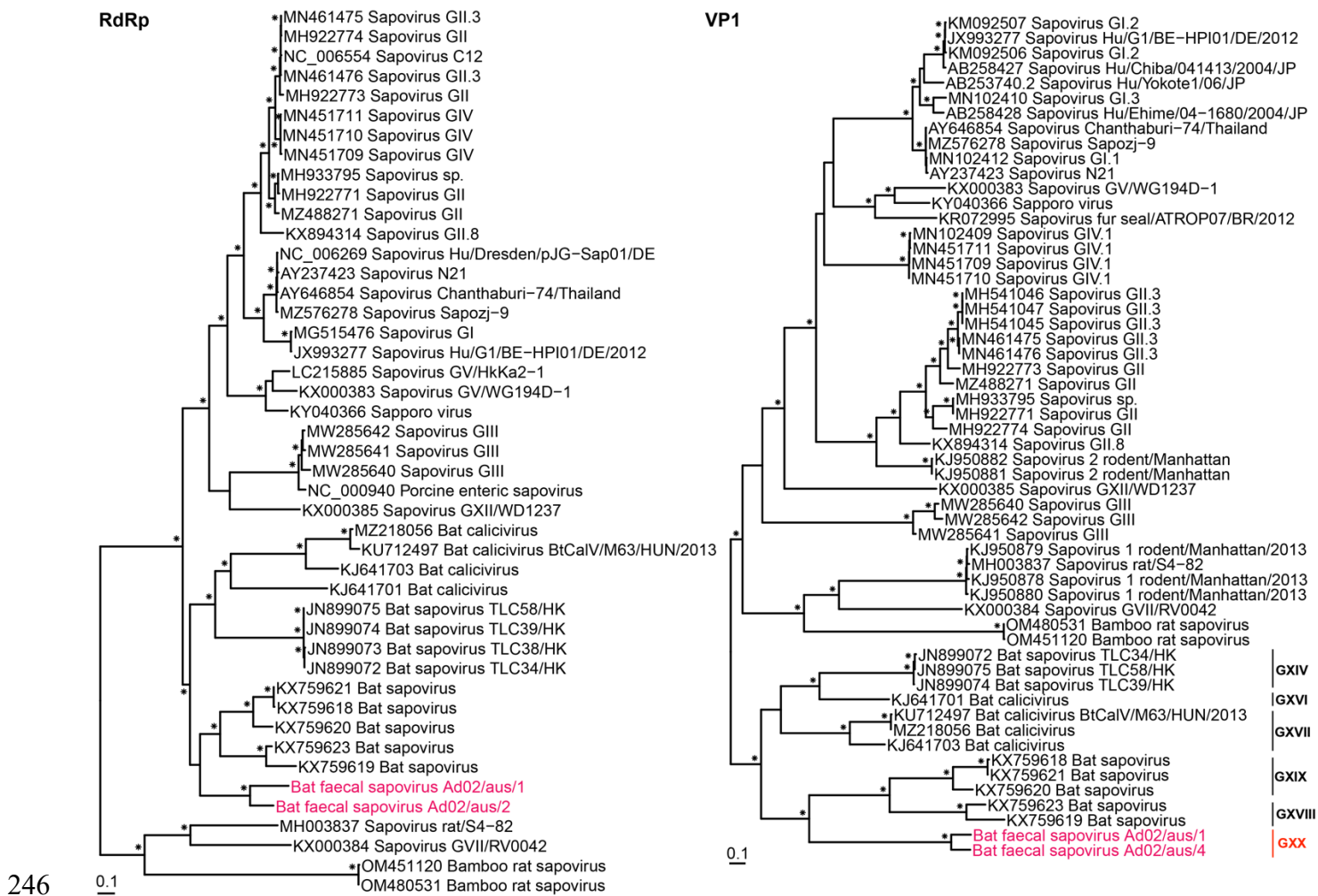
194

195 CP07/aus/1 contains ten ORFs in the arrangement ORF1a, ORF1ab, spike, NS3, envelope,
196 matrix, nucleocapsid, NS7a, NS7b and NS7c. Transcription Regulatory Sequences (TRS)
197 preceded all ORFs. Additional bat coronavirus contigs ranging from 318 to 1,309 bp were
198 detected in sequencing libraries from two Gordon sampling locations. These short contigs
199 shared 40-95% amino acid identity to CP07/aus/1. Three of these contigs contained RdRp or
200 spike amino acid sequences of sufficient length for phylogenetic analysis, and these were
201 provisionally denoted bat faecal coronavirus G05/aus/1, G05/aus/2 and G05/aus/3. Based on
202 phylogenetic analysis of the RNA-dependent RNA polymerase (RdRp) and/or spike protein,
203 the novel betacoronaviruses detected here fell within the *Betacoronavirus* subgenus
204 *Nobecovirus* (Fig. 2) and were most closely related to P.alecto/Aus/SEQ/2009 (for which
205 only a partial RdRp is available) sampled from a black flying fox in south east Queensland,
206 Australia (Smith et al., 2016) and to *Pteropus rufus nobecovirus* sampled from a flying fox in
207 Madagascar (accession no. OK067319; Fig. 2) (Kettenburg et al., 2022). CP07/aus/1 had
208 83% amino acid identity to *Pteropus rufus nobecovirus* over the complete ORF1ab replicase
209 and 97% to P.alecto/Aus/SEQ/2009 over the partial RdRp. Amino acid identity to *Pteropus*
210 *rufus nobecovirus* over the spike and non-structural proteins was 72% and 58%, respectively.
211 The RdRp of G05/aus/1 shared 95% amino acid identity to CP07/aus/1, while the partial
212 spike proteins of G05/aus/2 and G05/aus/3 shared 57% and 63% amino acid identity to

213 CP07/aus/1, respectively. It is possible that G05/aus/1 and G05/aus/2 represent transcripts
 214 from the same virus, while G05/aus/3 represents a different species to CP07/aus/1. However,
 215 this could not be confirmed as the G05/aus/3 genome was incomplete. Regardless, it is clear
 216 from the spike protein phylogeny that at least three different coronaviruses are circulating in
 217 the bats sampled here.



232 were detected in the same sequencing library. The nine sequences shared 66-74% nucleotide
233 and 76-81% amino acid identity to Ad02/aus/1 over the polyprotein, suggesting the presence
234 of additional diverse sapoviruses. The near complete Ad02/aus/1 genome is 7,254 bp and
235 contains two ORFs encoding a polyprotein (near complete with likely 45 residues missing
236 from the 5' end), and the VP2. Ad02/aus/1 exhibited 44.8% amino acid identity in the partial
237 polyprotein to its closest relative – Bat sapovirus Bat-SaV/Limbe65/CAM/2014 (accession
238 no. KX759620) – detected in the faeces of *Eidolon helvum* bats in Cameroon, Africa (Yinda
239 et al., 2017). Phylogenetic analysis of the RdRp and VP1 revealed a clustering of bat
240 sapoviruses in both trees that included the novel Australian bat sapoviruses found here (Fig.
241 3). Bat sapoviruses have been assigned to the putative genogroups GXIV, GXVI, GXVII,
242 GXVIII and GXIX based on VP1 phylogeny and amino acid sequence identities. Using the
243 same criteria, the novel sapovirus Ad02/aus/1 identified here should be assigned to its own
244 genogroup, putatively named GXX, which would also include the partial VP1 Ad02/aus/4
245 sequence (Supplementary Table 1, Fig. 3).



247 **Fig. 3.** Phylogenetic relationships of the novel bat sapoviruses using the amino acid
248 sequences of the RdRp and VP1. Amino acid alignment lengths were 491 and 623 residues
249 for the RdRp and VP1, respectively. Bat sapoviruses from this study are coloured by
250 sampling location (Adelaide – pink) and bootstrap values >70% are represented by the
251 symbol shown at the branch node. The putative bat sapovirus genogroups are displayed to the
252 right of the VP1 tree and our proposed putative genogroup is coloured in red. The trees are
253 rooted at midpoint for clarity and the scale bar represents the amino acid substitutions per
254 site.

255

256 **Novel birna-like virus (*Birnaviridae*)**

257 Sequences related to the *Birnaviridae* (double-stranded RNA viruses; dsRNA) were detected
258 in one Centennial Park and two Gordon libraries. All the birna-like virus sequences identified
259 in the Centennial Park and Gordon libraries shared >99% nucleotide identity, and the
260 complete coding region of segment B, which encodes the RdRp, was obtained from one
261 library (Gordon 05). The *Birnaviridae* segment A that encodes the polyprotein and a small
262 overlapping ORF was not identified in our data. Phylogenetic analysis revealed that the birna-
263 like virus RdRp sequence, denoted G05/aus/1, was most closely related (50% amino acid
264 identity) to the disease-causing virus Chicken proventricular necrosis virus (Fig. 4) (Guy et
265 al., 2011), forming a distinct clade that is distantly related to the birnaviruses that infect a
266 wide range of hosts.

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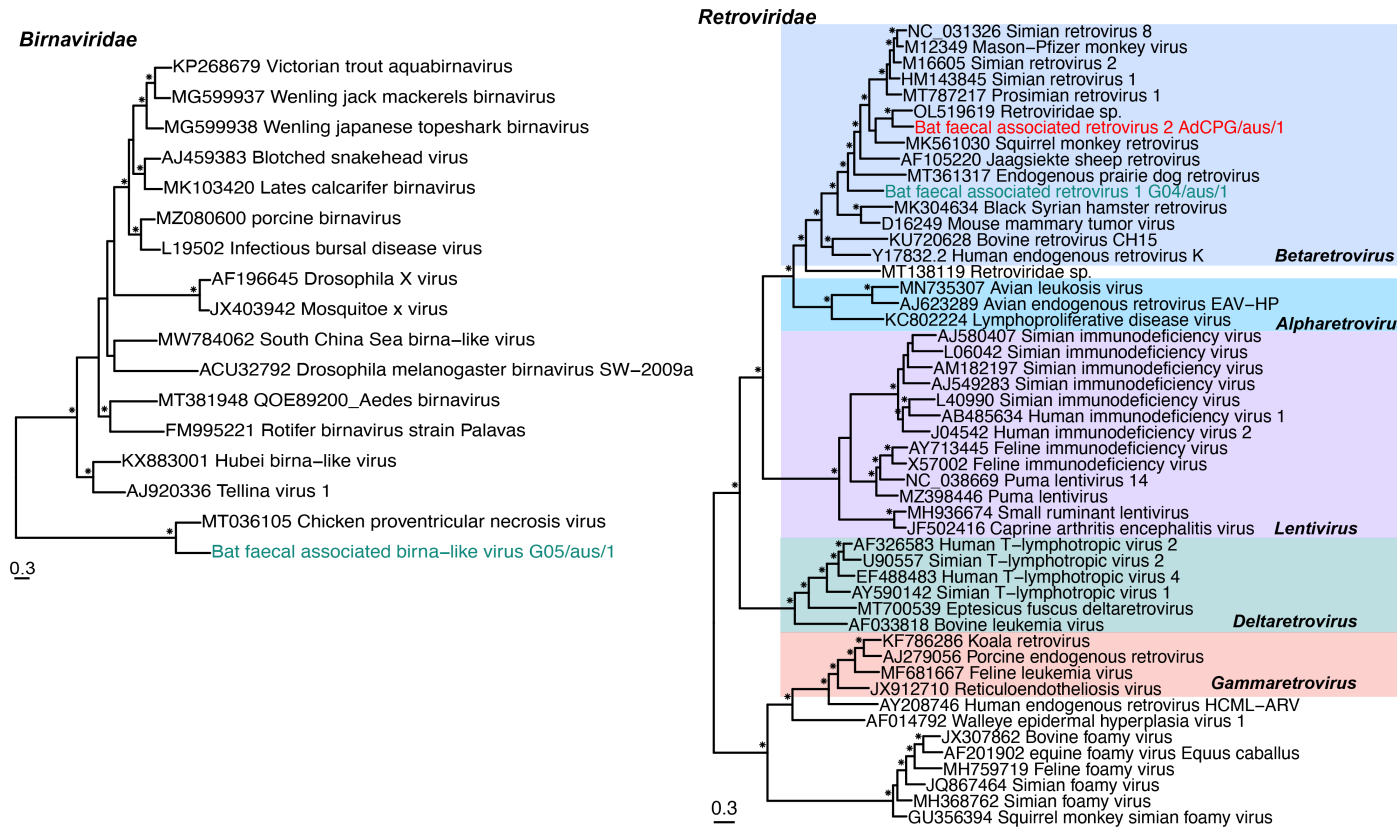
268 **Bat retrovirus (*Retroviridae*)**

269 A near complete genome of a retrovirus was identified in Gordon library 04 and provisionally
270 named bat faecal associated retrovirus 1 G04/aus/1. Four ORFs were observed over the 7,455
271 bp genome and assigned as the gag, pro, pol and env genes based on the presence of
272 conserved domains. In the pro gene we were able to identify an active site motif DTGAD
273 predominately observed in functional retroviruses, and a helix motif GRDVL (Turnbull and
274 Douville, 2018). We were unable to identify complete long terminal repeat (LTR) regions in
275 the 7,455 bp genome, although this may be due to incomplete assembly at the 5' and/or 3'
276 end, rather than a true absence of LTRs. Importantly, as the four ORFs contained the
277 appropriate retrovirus conserved domains and were uninterrupted by stop codons, it is
278 possible that G04/aus/1 is potentially exogenous and functional. A BLASTn analysis of the
279 complete G04/aus/1 genome revealed no match to any bat reference genome on
280 NCBI/GenBank. G04/aus/1 exhibited 56% amino acid identity in the pol protein to its closest

281 relative, Simian retrovirus 2 (accession M16605), a presumably exogenous retrovirus (Thayer
282 et al., 1987). The abundance for this novel retrovirus in the Gordon 04 library was 67 RPM
283 (2,457 reads) (Fig. 1C).

284

285 A further near complete retroviral genome was identified by reassembling 31 partial contig
286 sequences from 12 libraries from all three sample locations. This Bat faecal associated
287 retrovirus 2 AdCPG/aus/1 is 6,630 bp and contains four open reading frames encoding the
288 gag, pro, pol and env genes. It also contains the conserved domains expected in functional
289 retroviruses, although the terminal end of the env gene is missing (either from true truncation
290 or incomplete assembly). The virus is most closely related to AdCPG/aus/1 sampled from the
291 lung tissue of Malayan pangolins (Ning et al., 2022). BLASTn analysis of the complete
292 genome of AdCPG/aus/1 showed the absence of this genome in any bat reference genome on
293 NCBI/GenBank. AdCPG/aus/1 reads were detected in 13 libraries (two Adelaide, four
294 Centennial Park and seven Gordon) and the abundance in each library ranged from 3.7 – 68.8
295 RPM (127 – 1786 reads) (Fig. 1C). Phylogenetic analysis of the pol protein that contains the
296 reverse transcriptase (RT) domain revealed that G04/aus/1 and AdCPG/aus/1 fell within the
297 genus *Betaretrovirus*, clustering with both exogenous and endogenous retroviruses associated
298 with various mammalian species (Fig. 4).



299

300 **Fig. 4.** Phylogenetic analysis of the birna-like virus and bat retroviruses based on the RdRp
 301 and pol amino acid sequences, respectively. The *Birnaviridae* RdRp sequence alignment was
 302 767 amino acid residues in length while the *Retroviridae* pol alignment comprised 1,356
 303 residues. The viruses from this study are coloured by sampling location (Gordon – green) and
 304 the reassembled retrovirus sequence is in red (to indicate multiple locations). The
 305 *Retroviridae* genera are highlighted and bootstrap values >70% are represented by the symbol
 306 shown at the branch node. The tree is midpoint rooted for clarity, with the scale bar
 307 representing the amino acid substitutions per site.

308

309 **Invertebrate-associated viruses**

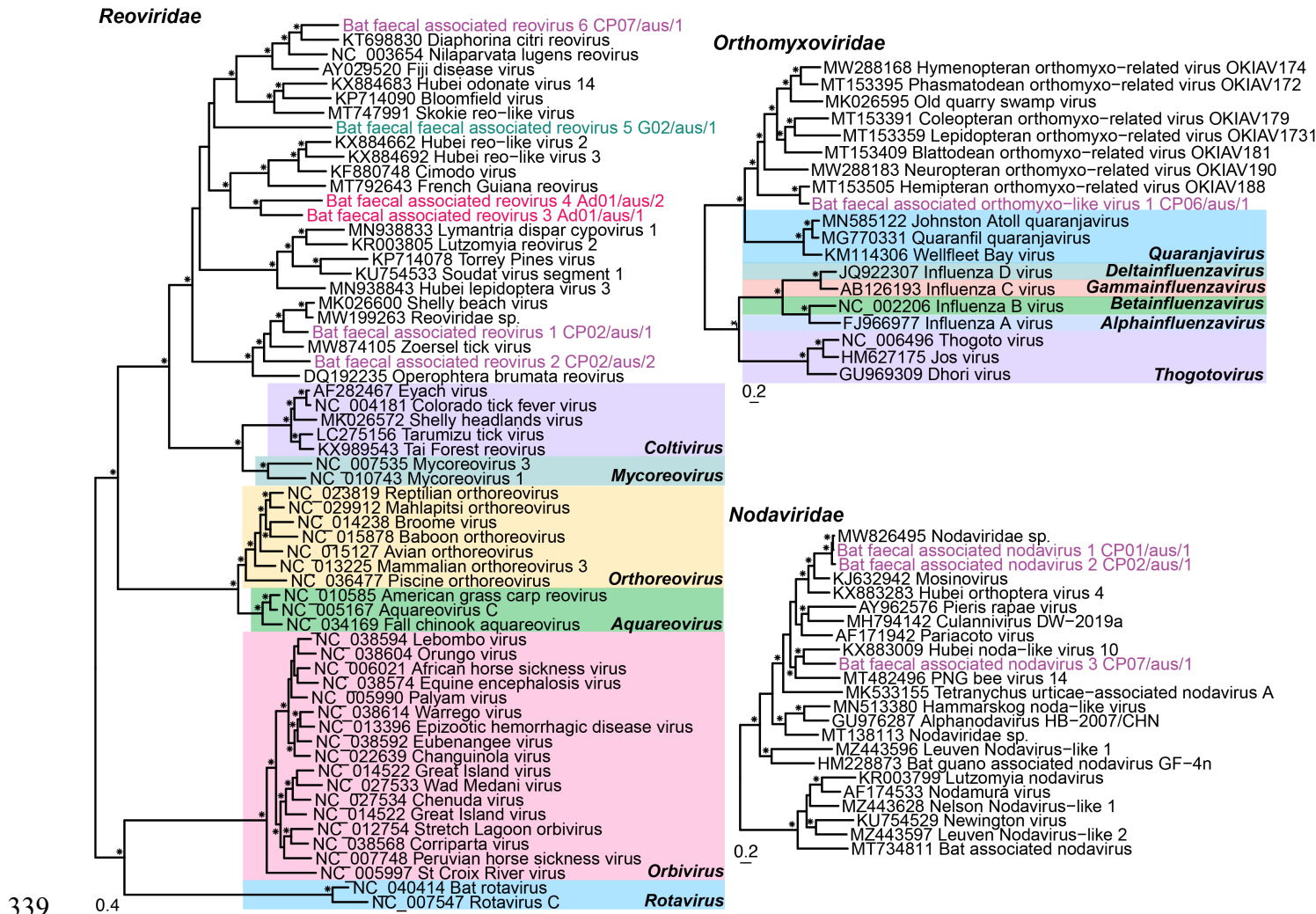
310 We detected likely invertebrate-associated virus sequences from seven single-strand
 311 negative-sense RNA viruses (-ssRNA), three +ssRNA virus and one dsRNA virus families, in
 312 addition to the order *Bunyavirales* (-ssRNA). The virus sequences from the *Chuviridae*,
 313 *Lispiviridae*, *Artoviridae*, *Nyamiviridae*, *Xinmoviridae*, *Qinviridae*, *Disctroviridae* and
 314 *Iflaviridae* are not discussed further, although information on positive libraries is provided
 315 (Supplementary Fig. 1) and phylogenetic analysis was performed (Supplementary Fig. 2).
 316 Virus sequences from the *Orthomyxoviridae*, *Nodaviridae*, *Reoviridae* and *Bunyavirales* are
 317 considered further as these viral groups include mammalian-infecting viruses, are important

318 vector-borne viruses, or are able to infect mammals experimentally (*Nodaviridae*, genus
319 *Alphanodavirus*).

320

321 Orthomyxovirus (-ssRNA virus) segments were identified in five libraries from Centennial
322 Park. Full coding regions for two polymerase segments – PB2 and PA – and the
323 hemagglutinin segment 2 and nucleocapsid segment 5 were present in all libraries, although a
324 full coding region for polymerase segment PB1 was only present in a single Centennial Park
325 library. The three polymerase proteins of Centennial Park library 06 were used for
326 phylogenetic analysis, which revealed that this sequence was most closely related to an
327 orthomyxovirus sampled from jumping plant lice in Australia (Fig. 5) (Käfer et al., 2019).

328 Nodaviruses (+ssRNA virus) were detected in five Centennial Park libraries and three
329 Gordon libraries. Both the RNA1 (RdRp) and RNA2 segments were identified, including two
330 sequences with the complete RdRp. Nodavirus CP01/aus/1 and CP02/aus/1 were related to a
331 nodavirus sampled from birds in China (Zhu et al., 2022) and most likely belong to the same
332 viral species, although these fragments were only 476 and 232 amino acids, respectively. The
333 nodavirus CP07/aus/1 RdRp segment was related to a nodavirus from arthropod hosts from
334 China (Fig. 5) (Shi et al., 2016). Gene segments related to the *Reoviridae* (dsRNA) were
335 present in all Centennial Park, three Gordon and one Adelaide library. The reovirus VP1 Pol
336 segments detected here were related, albeit distantly (~40% amino acid identity) to reoviruses
337 associated with ticks (Harvey et al., 2019, Vanmechelen et al., 2021), moths (Graham et al.,
338 2006), bat flies (Xu et al., 2022) and the Asian citrus psyllid (Nouri et al., 2015) (Fig. 5).



339

340 **Fig. 5.** Phylogenetic analysis of the invertebrate-associated reoviruses, orthomyxoviruses and

341 nodaviruses based on the VP1 Pol, concatenated PB2-PB1-PA and RdRp amino acid

342 sequences, respectively. Amino acid alignment length were 1,020 residues for *Reoviridae*,

343 2,233 residues for the *Orthomyxoviridae* and 774 residues for the *Nodaviridae*. Viruses from

344 this study are coloured by sampling location (Adelaide – pink, Centennial Park – purple and

345 Gordon – green) and genera are highlighted in the *Reoviridae* and *Orthomyxoviridae* trees.

346 Bootstrap values >70% are represented by the symbol shown at the branch node. The tree is

347 rooted at midpoint for clarity and the scale bar represents the amino acid substitutions per

348 site.

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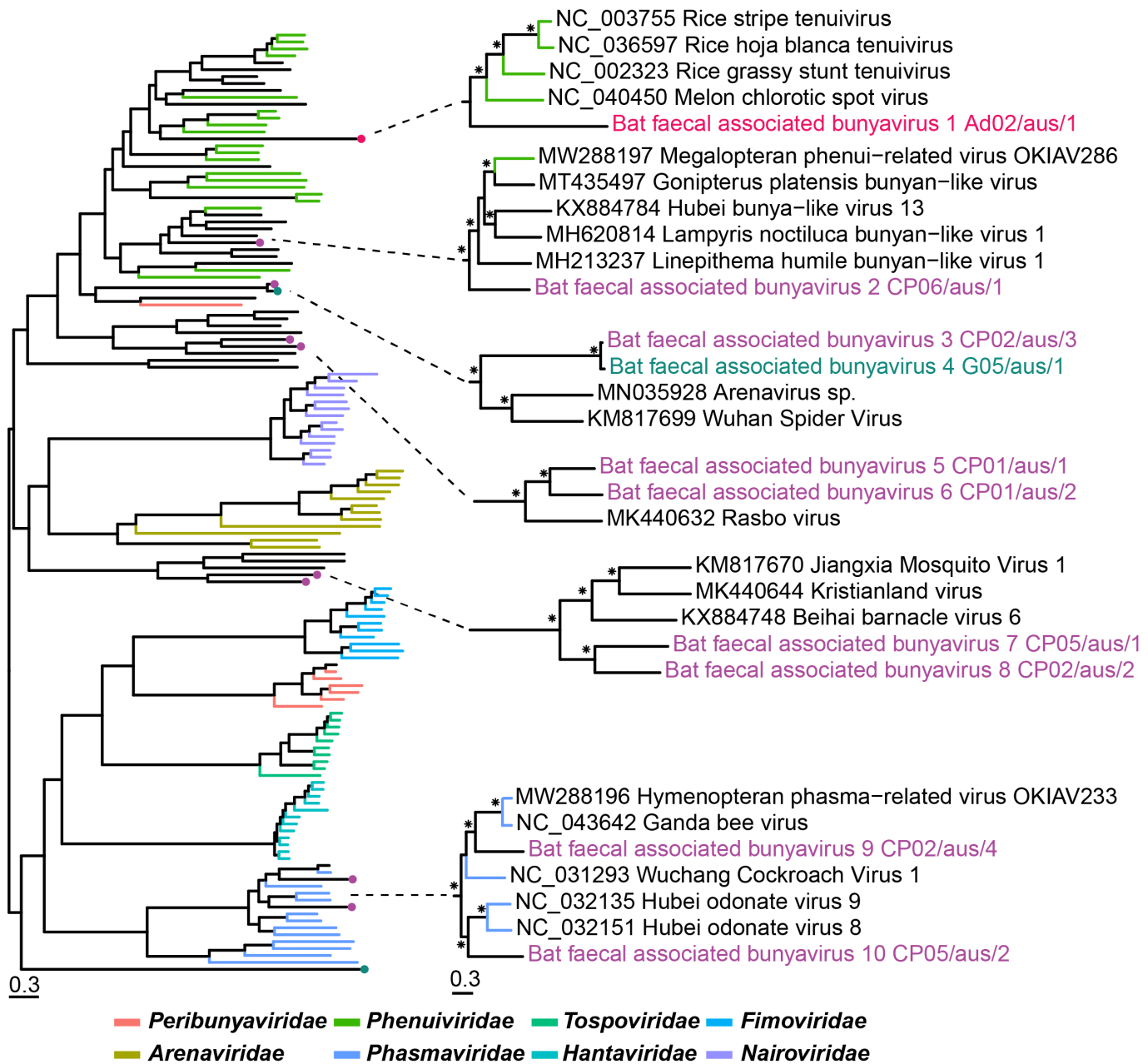
350 Finally, bunyavirus fragments were detected in all the Adelaide and Centennial Park libraries

351 and six Gordon libraries. Eleven RdRp coding regions were used for phylogenetic analysis

352 which revealed that two bunyavirus sequences fell into the *Phenuiviridae* and four were basal

353 to that family, while two sequences fell into the *Phasmaviridae*, two were basal to the

354 *Arenaviridae*, and one was basal to a grouping of five families (Fig. 6). The Adelaide
 355 bunyavirus Ad02/aus/1 was related to the plant associated genus *Tenuivirus* and the
 356 remaining 10 were related to invertebrate hosts (Fig. 6).



357
 358 **Fig. 6.** Phylogenetic analysis of viruses from the order *Bunyvirales*. The RdRp amino acid
 359 sequence was used to estimate phylogenetic trees and the alignment length was 1,434 amino
 360 acid residues. Viruses from this study are coloured by sampling location and bootstrap values
 361 >70% are represented by the symbol shown at the branch node. The tree is midpoint rooted
 362 for clarity and the scale bar represents the amino acid substitutions per site.

363

364 4. Discussion

365 Virological surveillance of bats in Australia has largely focused on screening for known
366 zoonotic viruses such as Hendra virus and Australian bat lyssavirus, although the
367 paramyxovirus Tioman virus, for which flying foxes are the natural host, and coronaviruses
368 are also targeted (Boardman et al., 2020, Prada et al., 2019a, Smith et al., 2016). The primary
369 aim of these studies is to identify specific viruses using either PCR or serological data.
370 Although such surveillance has been successful in determining the active circulation of these
371 specific viruses, these approaches necessarily have restricted capacity to detect novel or
372 unexpected viruses, thus providing a limited understanding of viruses circulating in
373 Australian bats. As bats are frequently found near human populations, they are of particular
374 concern regarding potential zoonoses (Plowright et al., 2011, Williams et al., 2006, Halpin et
375 al., 2000). Herein, we used metatranscriptomics to reveal the natural faecal virome of the
376 grey-headed flying fox. Although most of the viruses identified were likely associated with
377 bat diet, as expected from faecal sampling, we also identified viruses from three mammalian-
378 associated families (*Coronaviridae*, *Caliciviridae*, *Retroviridae*) and one virus from the
379 *Birnaviridae* family that may also have a mammalian association.

380

381 Both alpha- and betacoronaviruses have been identified in a variety of bat species (Smith et
382 al., 2016, Prada et al., 2019b). Here, we characterised the complete genome of a
383 betacoronavirus in grey-headed flying foxes that was closely related to two other
384 betacoronaviruses sampled in flying foxes in Australia and Madagascar (Smith et al., 2016,
385 Kettenburg et al., 2022). The current ICTV classification for coronavirus species states that
386 less than 90% amino acid identity in the ORF1ab conserved replicase domains constitutes a
387 new species. Although bat faecal coronavirus CP07/aus/1 shares high sequence similarity to
388 another reported bat betacoronavirus, the P.alecto/Aus/SEQ/2009 sequence is only 146 amino
389 acids in length, does not span the complete RdRp and is therefore difficult to classify.
390 Accordingly, we suggest that betacoronavirus bat faecal coronavirus CP07/aus/1 represents a
391 novel species, to which P.alecto/Aus/SEQ/2009 may also belong. The complete genome of
392 this virus was found in both Adelaide and New South Wales (99.8% nucleotide similarity
393 between the two genomes) and abundance counts were high in both locations (Fig. 1C),
394 indicative of virus exchange between bat populations. Flying foxes are known to travel long
395 distances to feed, roosting sites change depending on season, and in Australia several flying
396 fox species share roosting sites (Timmiss et al., 2021), all of which provide opportunities for
397 viruses to infect new individuals. Importantly, while we were only able to assemble the
398 complete genome of one novel coronavirus, we identified partial genome fragments of at

399 least two more diverse coronaviruses (Fig. 2), indicating that Australian bats carry a high
400 diversity of coronaviruses as has been seen in other bat species.

401

402 This is the first report of a sapovirus in Australian bats. Previously, bat sapoviruses have been
403 sampled from *Eidolon helvum* (Straw-coloured fruit bat) in Cameroon (Yinda et al., 2017)
404 and Saudi Arabia (Mishra et al., 2019) and *Hipposideros Pomona* (Pomona leaf-nosed bat)
405 from Hong Kong (Tse et al., 2012). Currently, the bat sapoviruses characterised have been
406 from bats with no apparent disease (Tse et al., 2012, Yinda et al., 2017, Mishra et al., 2019).
407 Whether this is the case here is unknown because the reliance on faecal sampling meant that
408 there was no direct interaction with individual animals. The disease potential of bat
409 sapoviruses should be investigated further as sapoviruses have been linked to acute
410 gastroenteritis outbreaks in humans (Oka et al., 2015) and some animal sapoviruses are
411 closely related to those found in humans (Mombo et al., 2014, Firth et al., 2014, Martella et
412 al., 2008).

413

414 Until the metagenomic detection of porcine birnavirus (Yang et al., 2021) and porcupine
415 birnavirus (He et al., 2022) it was believed the *Birnaviridae* infected fish, insects and birds
416 exclusively (Crane et al., 2000, Da Costa et al., 2003, Chung et al., 1996, Brown and Skinner,
417 1996, Guy et al., 2011). We identified the segment B sequence of a novel bat faecal
418 associated birna-like virus that was most closely related to a divergent pathogenic avian
419 birnavirus (50% amino acid identity). Given its divergent phylogenetic position it is currently
420 unclear whether this virus actively infects grey-headed flying foxes or is associated with a
421 component of their diet or microbiome. While grey-headed flying foxes are not insectivores,
422 the ingestion of insects through the consumption of fruit and nectar seems likely given the
423 high number of invertebrate, plant and fungi viruses sequenced here (Fig. 1B, Supplementary
424 Fig. 1). The moderate abundance values (30.5 and 19.9 RPM) cannot exclude either scenario
425 as using a host reference gene such as COX1 for sequencing depth comparison may not be as
426 reliable for faecal samples as it would be when analysing tissue. Further investigation is
427 needed to determine the natural host of bat faecal associated birna-like virus and to determine
428 what tissue types are affected.

429

430 Two intact, possibly exogenous retrovirus near complete genomes were also identified in this
431 study and were most closely related to mammalian infecting retroviruses from the genus
432 *Betaretrovirus*. Six retroviruses have been previously characterised from Australian bat brain

433 tissue and excretions (including faeces), all from the genus *Gammaretrovirus* (Hayward et
434 al., 2020, Cui et al., 2012) and hence highly divergent from the viruses identified here.
435 Although the exogenous status needs to be confirmed, it is possible that bat faecal associated
436 retrovirus 1 G04/aus/1 and bat faecal associated retrovirus 2 AdCPG/aus/1 constitute the first
437 exogenous and intact betaretroviruses sampled from the faeces of bats in Australia.
438 Unfortunately, virus identification through metatranscriptomics does not provide reliable
439 information on whether a virus is endogenous and defective, or still functional and exogenous
440 (Hayward et al., 2013, Hayward and Tachedjian, 2021). That the retroviruses detected here
441 have all the necessary genes to comprise a functional virus, with undisrupted ORFs, were not
442 detected in every library, and are not present in the bat genome, at the very least suggests that
443 they are only recently endogenized and currently unfixated in the bat population. Further work
444 confirming the nature of the retroviruses detected here is warranted since bats are known to
445 be major hosts for retroviruses (Cui et al., 2015) and their cross-species transmission across
446 mammalian orders is commonplace (Hayward et al., 2013).

447

448 In addition to mammalian viruses, we detected virus sequences that are likely invertebrate-
449 associated. Of particular interest were those from the *Orthomyxoviridae* and *Reoviridae* that
450 span a wide variety of hosts including mammals and were at high abundance in some of the
451 Centennial Park libraries. Notably, bat faecal associated reovirus 1 CP02/aus/1 groups with
452 members of the *Reoviridae* associated with ticks that are vectors for numerous pathogenic
453 microorganisms, particularly from the genus *Coltivirus* – Colorado tick fever virus and Eyach
454 virus (Goodpasture et al., 1978, Rehse-Küpper et al., 1976). Additionally, a novel coltivirus –
455 Tai Forest reovirus – was sampled from bats in Cote d’Ivoire and shown to infect human cells
456 (Weiss et al., 2017). The current evidence for tick-borne reovirus infection in humans
457 highlights the importance of assessing the pathogenic potential of new tick associated
458 reoviruses, especially those viruses discovered in urban wildlife.

459

460 Our study highlights the diversity of viruses in wildlife species from metropolitan areas. In
461 this context it is notable that the bat coronaviruses identified fall within the subgenus
462 *Nobecovirus* of betacoronaviruses. Currently, this subgenus is strongly associated with bats
463 sampled on multiple continents, with the phylogenetic depth of the *Nobecovirus* lineage
464 further suggesting that bats have harbored these viruses for millennia with no apparent
465 infection of humans. Hence, although the bats studied were resident in urban/suburban
466 locations, this does not necessarily translate into a clear risk of human emergence.

467

468 **Data statement**

469 The raw data generated for this study are available in the NCBI SRA database under the
470 BioProject accession number PRJNA851532 and SRA accession numbers SRR19790899-
471 SRR19790914. All genome sequences presented in phylogenetic trees are available in NCBI
472 GenBank under the accession numbers ON872523-ON872588.

473

474 **Declaration of interests**

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

477

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479

480 **Ethics statement**

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483

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487

488 **CRedit authorship contribution statement**

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499

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