

1 **Global genetic patterns reveal host tropism versus cross-taxon transmission of bat**
2 **Betacoronaviruses**

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17 **Abstract**

18 Emerging infectious diseases due to coronavirus (CoV) infections have received significant global
19 attention in the past decade and have been linked to bats as the original source. The diversity, distribution,
20 and host associations of bat CoVs were investigated to assess their potential for zoonotic transmission.
21 Phylogenetic, network, and principal coordinate analysis confirmed the classification of betacoronaviruses
22 (BetaCoVs) into five groups (2A to 2E) and a potentially novel group, with further division of 2D into five
23 subgroups. The genetic co-clustering of BetaCoVs among closely related bats reflects host taxon-
24 specificity with each bat family as the host for a specific BetaCoV group, potentially a natural barrier
25 against random transmission. The divergent pathway of BetaCoV and host evolution suggests that the
26 viruses were introduced just prior to bat dispersal and speciation. As such, deviant patterns were
27 observed such as for 2D-IV, wherein cross-taxon transmission due to overlap in bat habitats and
28 geographic range among genetically divergent African bat hosts could have played a strong role on their
29 shared CoV lineages. In fact, a few bat taxa especially the subfamily Pteropodinae were shown to host
30 diverse groups of BetaCoVs. Therefore, ecological imbalances that disturb bat distribution may lead to
31 loss of host specificity through cross-taxon transmission and multi-CoV infection. Hence, initiatives that

32 minimize the destruction of wildlife habitats and limit wildlife-livestock-human interfaces are encouraged to
33 help maintain the natural state of bat BetaCoVs in the wild.

34

35 **Key Words:** Coronaviruses, Bats, Phylogeny

36

37 **Importance**

38 Bat Betacoronaviruses (BetaCoVs) pose a significant threat to global public health and have been
39 implicated in several epidemics such as the recent pandemic by severe acute respiratory syndrome
40 coronavirus 2. Here, we show that bat BetaCoVs are predominantly host-specific, which could be a
41 natural barrier against infection of other host types. However, a strong overlap in bat habitat and
42 geographic range may facilitate viral transmission to unrelated hosts, and a few bat families have already
43 been shown to host multi-CoV variants. We predict that continued disturbances on the ecological balance
44 may eventually lead to loss of host specificity. When combined with enhanced wildlife-livestock-human
45 interfaces, spillover to humans may be further facilitated. We should therefore start to define the
46 ecological mechanisms surrounding zoonotic events. Global surveillance should be expanded and
47 strengthened to assess the complete picture of bat coronavirus diversity and distribution and their
48 potential to cause spillover infections.

49

50 **Introduction**

51 Emerging and re-emerging infectious diseases greatly affect public health and global economies
52 (1). These diseases involve pathogenic strains that recently evolved, pathogens that infect human
53 population for the first time, and pathogens that re-occur at higher frequency (2). Majority of these
54 emerging infectious diseases are caused by microorganisms from non-human source or zoonotic
55 pathogens from wild animals (3). In particular, emerging infectious diseases due to coronavirus (CoV)
56 infections have been receiving significant global attention as exemplified by the severe acute respiratory
57 syndrome coronavirus (SARS-CoV) outbreak in 2002-2003, the Middle East respiratory syndrome
58 coronavirus (MERS CoV) outbreak in 2012, and the recent SARS-CoV2 pandemic which causes the

59 Coronavirus Disease of 2019 (COVID-19), all of which have been linked to bats as the original source (4-
60 7).

61 Coronavirus (CoVs) are pleiomorphic, single-stranded positive-sense RNA viruses with three
62 major structural proteins; a nucleocapsid protein (N) which functions in encapsidating genomic RNA and
63 facilitating its incorporation into virions, a small integral membrane protein (M) with intrinsic membrane-
64 bending properties that plays a central role in viral assembly, an envelope glycoprotein (E), and a large
65 spike protein (S) which functions in viral entry and pathogenesis (8-11). CoVs are considered to have the
66 largest genome among RNA viruses at approximately 27 to 30 kb (6). There are four genera by which
67 CoVs are classified, namely Alphacoronaviruses, Betacoronaviruses, Gammacoronaviruses and
68 Deltacoronaviruses (12). Betacoronaviruses (BetaCoVs) are of particular importance as the SARS-CoV,
69 MERS-CoV, and SARS-CoV2 which have caused global epidemics belong to this lineage (5).
70 Betacoronaviruses (BetaCoVs) are further classified into subgenera Embecovirus (also lineage 2A and
71 includes Murine CoV and ChRCoV HKU24), Sarbecovirus (lineage 2B and includes SARS-related CoVs),
72 Merbecovirus (lineage 2C and includes Ty-BatCoV HKU4, Pi-BatCoV HKU5, Hp-BatCoV HKU25, and
73 MERS-related CoVs), Nobecovirus (lineage 2D and includes Ro-BatCoV HKU9 and Ro-BatCoV
74 GCCDC1), and Hibecovirus (lineage 2E and includes Bat Hp-betaCoV Zhejiang2013) (13).

75 HCoV-229E and HCoV-OC43, both human coronaviruses (HCoVs), were first discovered in
76 patients with mild respiratory illness (14). Two new species of HCoVs, the HCoV-NL63 and HCoV-HKU1
77 were also discovered in 2004 and 2005, respectively (15). Disease types caused by HCoVs usually range
78 from gastrointestinal infections, upper respiratory infections, and lower respiratory infections such as
79 pneumonia (16). Further studies revealed that CoVs cause respiratory, enteric, hepatic and neurological
80 diseases in animals like bats, birds, cats, dogs, pigs, mice, horses, and whales (17). Moreover, the
81 SARS-CoV and MERS-CoV which have clear zoonotic origins have also been found to cause lower
82 respiratory infections such as pneumonia. In particular, SARS-CoV has been associated with diffuse
83 alveolar damage (DAD) and acute respiratory distress syndrome (ARDS), while MERS-CoV has been
84 linked to renal failure (16). Recently, SARS-CoV2 has been reported to cause pneumonia, ARDS and
85 multiple organ failure (7,18). CoVs can be transmitted via fecal-oral route, respiratory, as well as contact
86 transmission (19). The spread of SARS-CoV has been primarily attributed to human-human transmission

87 via direct contact with respiratory droplets and exposure to fomites (20). Similarly, although no evidence
88 of being sustained, human-human transmission has also been reported for MERS-CoV infections (21).
89 The World Health Organization (WHO) released guidelines on how to limit human-human transmission,
90 and reduce the risk of animal-human transmission in order to contain the rapidly spreading COVID-19
91 disease that started in Wuhan, China (22).

92 There is increasing evidence for the role of bats as hosts of emerging pathogens, specifically
93 viruses (23). Bats (Order: Chiroptera) are one of the most diverse and widely distributed animals, second
94 only to rodents as the most speciose order in class Mammalia (24). They are classified into two suborders,
95 Yinpterochiroptera, which include the false vampire bats (Megadermatidae), horseshoe bats
96 (Rhinolophidae), and megabats or fruit bats (Pteropodidae); and Yangochiroptera which includes vesper
97 bats (Vespertilionidae), sac-winged bats (Emballonuridae) and bulldog bats (Noctilionidae) (25). A
98 significant number of CoVs can be found in bats, thus future spill-over events presents a constant threat
99 to global health (26). In particular, emerging human coronaviruses have been linked to bat sources.
100 Although camels were the source of the MERS-CoV in the Middle East, a coronavirus with 100% nt
101 identity to that of human β -CoV 2c EMC/2012 isolated from a case-patient has been found in bats at a
102 detection rate of 3.5% (27). SARS-like coronaviruses with 92% identity to that of human SARS-CoV
103 isolates have also been detected in horseshoe bats in China. Three species of horseshoe bats from
104 China namely *Rhinolophus pearsoni*, *R. pussilus* and *R. macrotis* demonstrated 28%, 33% and 78%
105 SARS-CoV seroprevalence, respectively (28). Furthermore, SARS-CoV2 was found to be 96% identical
106 to a bat coronavirus at the whole genome level (29). Bat CoVs (BtCoVs) comprise only 6% of the current
107 CoV database although roughly 3,000 genetic lineages of BtCoVs are believed to circulate worldwide (30).
108 It has also been suggested that future CoV outbreaks can be geographically predicted based on the
109 specific bat species distribution (12). These highlight the need to expand our knowledge on BtCoVs,
110 particularly on their diversity, distribution, host association, and evolution to understand their potential for
111 zoonotic transmission. In this study, these parameters were assessed by classifying representative and
112 unresolved BetaCoVs based on network and phylogenetic analysis of their RNA-dependent RNA
113 polymerase sequences and evaluating patterns of geographic and host distribution. The analysis
114 validated the current classification scheme of BetaCoVs with potentially novel groupings and

115 subgroupings identified. Comparative phylogenetics demonstrated a strong tendency towards host
116 specificity of bat BetaCoVs although there was poor evidence of co-evolution with their hosts.

117

118 **Results**

119 **CoV detection in fruit bats from Southern Philippines**

120 Small and large intestine samples were collected from 49 bat individuals, 67.35% of which belong
121 to the lesser dog-faced fruit bat *Cynopterus brachyotis* mostly from residential sites but also present in the
122 agricultural and forest sites, 20.41% to *Rousettus amplexicaudatus* all from agricultural sites, and 10.2%
123 to the long-tongued nectar bat *Macroglossus minimus* mostly collected from agricultural sites (Table 1).
124 Only one (2.04%) cave nectar bat *Eonycteris spelaea* was collected, which was captured in a forest site.
125 Out of the 49 fruit bats tested, seven (14.29%) were positive for BtCoV based on RT-nPCR detection, all
126 of which were from the bat species *C. brachyotis* (Table 1). The species-level detection rate of CoV
127 among the *C. brachyotis* samples was 21.2% (7 out of 33), with five individuals positive for the small
128 intestine samples, and two other individuals positive for the large intestine samples (Table 1). Most of the
129 CoV-positive bats were females and juveniles that were captured in residential and forest sites near a
130 watershed reservation (supplemental data).

131

132 **Phylogenetic relationships of global CoVs**

133 A phylogenetic tree based on the partial RdRp gene sequences of CoVs obtained from this study
134 and those mined from the NCBI database representing CoVs from various bat species, domestic and wild
135 animals, as well as MERS-CoV, SARS-CoV and the SARS-CoV2, all from human patients was generated
136 (Figure 1). Alpha and BetaCoVs each formed a distinct lineage from a common ancestor. Based on
137 reference sequences, BetaCoVs diverged to five major phylogenetic clades classified as 2A
138 (Embecovirus), 2B (Sarbecovirus), 2C (Merbecovirus), 2D (Nobecovirus), the recently proposed 2E
139 (Hibecovirus) (13), and an unresolved clade which formed a genetic cluster distinct from the rest of the
140 currently recognized BetaCoV subgroups. The same CoV groupings were supported by the network
141 analysis using median-joining (Fig. 2A) and principal coordinate analysis using the distance matrix (Fig.
142 2B), wherein sequences from each clade and sub-clade formed corresponding unique and distinct

143 clusters. Clade 2A was composed of human CoV (HCoV-OC43), porcine CoV (JL/2008), BtCoV
144 (KX285045), and cattle CoV (NC_003045). Clade 2B was composed of human SARS-CoV, SARS-CoV2,
145 SARS-like CoVs, and unclassified BtCoVs, while clade 2C was composed of human MERS-CoV, camel
146 MERS-like CoVs, BtCoVs such as HKU5-1, and unclassified BtCoVs. BtCoVs such as HKU9 and
147 GCCDC1 formed clade 2D along with many unclassified BtCoVs, which further diverged into five distinct
148 subgroupings 2D-I to 2D-V. Clade 2E was composed of Bat HP-BetaCoV/Zhejiang2013 which is currently
149 the only recognized strain that belongs to the subgenus Hibecovirus (13). Finally, the unresolved clade is
150 composed of unclassified BtCoVs. The phylogenetic tree captured the current classification scheme for
151 BetaCoVs with novel information on Nobecovirus subclassification. However, deviant samples were also
152 observed such as BetaCoVs from *Rhinolophus pusillus* and *Myotis dasycneme* that clustered with the
153 AlphaCoV lineage as consistently demonstrated by the phylogenetic, network, and principal coordinate
154 analyses (Fig. 1 and 2).

155

156 **Geographical distribution of bat CoVs**

157 The regional distribution of bat CoVs comprising the major clades and subclades were examined
158 using the network analysis as shown in Figure 3 and Table 2. Results showed a heterogeneous
159 geographical distribution of CoVs for most of the clades, except for the 2D-II and 2D-IV subclade, which
160 were exclusively found in bats from Southeast Asia and Africa, respectively. In contrast, Clade 2B or the
161 Sarbecoviruses was distributed in Europe, East Asia, Southeast Asia, and Australia. Clade 2C or the
162 Merbecoviruses also had regionally diverse distribution in East Asia, Middle East, Europe, Africa, and
163 South America. The fruit bat subclade 2D-I also has a wide distribution from Africa, East Asia, and
164 Southeast Asia, 2D-III in East and Southeast Asia, and 2D-V in South and Southeast Asia. The single
165 representative CoV in 2E was from East Asia. The mammalian CoVs in 2A were from East Asia,
166 Southeast Asia and North America, while the unresolved clade consisted of unclassified BtCoVs from
167 South America.

168

169 **Bat hosts of BetaCoV groups**

170 The bat hosts were further evaluated to determine common patterns within the BetaCoV lineages.
171 Network analysis revealed a heterogenous composition in most of the clades or subclades in terms of the
172 bat source (Fig. 4 and Table 2). Clade 2A included one BtCoV from *Pteropus alecto*. Clade 2B, which
173 includes the human SARS-CoV and SARS-CoV2, was primarily composed of BtCoVs from horseshoe
174 bats (family Rhinolophidae) belonging to *Rhinolophus* sp. (67%), along with some Old World leaf-nosed
175 bats (family Hipposideridae) such as *Rhinonictis aurantia* and *Hipposideros galeritus*. Clade 2C of
176 MERS-CoV was primarily composed of vesper bat hosts (family Vespertilionidae) such as *Pipistrellus* sp.,
177 *Neoromicia capensis*, *Hypsugo savii*, *Nyctalus noctula*, *Eptesicus* sp., and *Vespertilio sinensis*, wherein
178 majority were sampled from *Pipistrellus* (46%). A CoV from *Eumops glaucinus* of the free-tailed bats
179 (family Molossidae) was also found to cluster with this group. Meanwhile, clade 2D consisted primarily of
180 fruit bat hosts (family Pteropodidae) and showed distinct subgroupings. CoVs from subfamilies
181 Rousettinae (*Rousettus* sp. and *Eonycteris spelaea*), Pteropodinae (*Dobsonia* sp.), and Macroglossini
182 (*Macroglossus minimus*) formed the subclade 2D-I and 2D-III, majority of which were sampled from the
183 genus *Rousettus* (67%). CoVs from subfamilies Cynopterinae (*Cynopterus* sp., *Dyacopterus spadiceus*,
184 *Megaerops niphanae*, and *Ptenochirus jagori*), and Macroglossini (*Macroglossus minimus*) formed
185 subclade 2D-II, with the genus *Cynopterus* (83%) as the predominantly sampled group. 2D-IV was
186 composed of CoVs mostly sampled from the African bat *Eidolon helvum* (subfamily Pteropodinae) (32%),
187 and the rest from other African fruit bats of subfamily Epomophorinae (*Micropteropus pusillus*,
188 *Epomophorus* sp., *Epomops franqueti*, *Megaloglossus woermanni*, and *Mynonycteris angolensis*),
189 subfamily Rousettinae (*R. aegyptiacus*), and family Hipposideridae (*Triaenops persicus*). Subclade 2D-V
190 was composed solely of CoVs from *Pteropus* sp., commonly known as flying foxes (subfamily
191 Pteropodinae), while the sole BtCoV representative in clade 2E has been detected in *Hipposideros pratti*
192 (family Hipposideridae). Finally, the unresolved clade was composed of American leafed (family
193 Phyllostomidae) and mustached (family Mormoopidae) bat hosts.

194

195 **Comparative phylogenetics of BetaCoVs and their bat hosts**

196 Phylogenetic analysis using the *cytB* gene was subsequently conducted to understand the
197 evolutionary relationships of the bat hosts within BetaCoV lineages (Fig. 5A). The microbats (suborder

198 Yangochiroptera) formed two distinct lineages: the vesper bats versus the American leafed, mustached,
199 and free-tailed bats. The megabats (suborder Yinpterochiroptera) also formed lineages corresponding to
200 the three families: horseshoe, Old World leaf-nosed, and fruit bats. Furthermore, the fruit bats were sub-
201 divided accordingly into Macroglossini, Pteropodinae, Cynopterinae, Rousettinae, and Epomophorinae
202 lineages, except for *E. helvum* which was separated from the Pteropodinae group.

203 In general, there was a genetic clustering of bat hosts found within BetaCoV lineages. The 2B
204 BetaCoVs was comprised of horseshoe and Old World leaf-nosed bat hosts that shared a common
205 ancestor. A similar pattern was observed for 2C and the 2D subgroups. The 2C BetaCoVs was composed
206 of vesper bat hosts, 2D-I/2D-III of Rousettinae bat hosts, 2D-II of Cynopterinae bat hosts, and 2D-IV of
207 Epomophorinae bat hosts, wherein each bat group also formed their corresponding genetic clade. The
208 2D-V subgroup was composed of solely *Pteropus* sp. which belongs to Pteropodinae. Finally, the
209 unresolved CoV clade was represented by American leafed and mustached microbat hosts (*P. davysii* and
210 *A. lituratus*) that clustered with a common ancestor. Meanwhile, 2E was represented by only one bat host:
211 *Hipposideros* sp. of the Old World leaf nosed bats.

212 However, some deviations were also noted. For example, bat hosts that belong to genetically
213 unrelated taxa were mixed in some BetaCoV groups. The Mollosidae bat *Eumops glaucinus* was found in
214 2C BetaCoV of vesper bats, the Pteropodinae *Dobsonia moluccensis* in 2D-I of Rousettinae bats, the
215 Pteropodinae *Macroglossus minimus* both in 2D-II of Cynopterinae bats and 2D-III of Rousettinae bats,
216 and the Pteropodinae *Eidolon helvum*, Rousettinae *Rousettus aegyptiacus*, and Hipposideridae/Old
217 World leaf-nosed bat *Trianeops persicus* in 2D-IV of Epomophorinae bats. Looking at the host, certain bat
218 families were observed to harbor BetaCoVs that belong to various lineages. The Rousettinae bats were
219 found to carry both 2D-I/2D-III and 2D-IV BetaCoVs, and the Old World fruit bats 2B, 2D-IV, and 2E. The
220 subfamily Pteropodinae also hosted Nobecoviruses from various groups such as 2A (*P. alecto*), 2D-I (*D.*
221 *moluccensis*), 2D-II (*D. spadiceus* and *M. minimus*), 2D-III (*M. minimus*), 2D-IV (*E. helvum*), and 2D-V
222 (*Pteropus* sp.).

223 The divergence of the bats and their BetaCoVs were compared to evaluate common evolutionary
224 pathways (Fig. 5B). The vesper microbats diverged as a separate group from the rest of the bats. The
225 remaining bats further diverged into different clades: the first one comprised of the other microbat families

226 (American leafed, mustached and free-tailed bats), and the second clade splitting into horseshoe bats,
227 Old World leaf-nosed bats, and fruit bats, with the former two sharing a much recent common ancestor.
228 For the corresponding viruses however, the evolutionary pattern was different. The unresolved microbat
229 CoVs were the first to diverge from the rest of the bat BetaCoVs. The vesper microbat CoVs (2C) on the
230 other hand appear to have diverged together with the Old World leaf-nosed megabat CoVs (2E). Finally,
231 the mammalian CoVs (2A) emerged from a lineage of bat BetaCoVs.

232

233 **Discussion**

234 A global analysis was conducted for BetaCoVs of human, animal, and bat origins including a
235 complete set of representatives from the updated CoV classification (13). The major clades inferred from
236 the generated Bayesian phylogenetic tree using partial RdRp sequences was consistent with previously
237 reported classification of BetaCoVs (2A or Embecoviruses, 2B or Sarbecoviruses, 2C or Merbecoviruses,
238 2D or Nobecoviruses, and 2E or Hibecoviruses) using whole genome sequences of representative strains
239 (12) and was able to position formerly unclassified bat CoVs. A new clade of unclassified bat BetaCoVs
240 that is genetically distinct from the currently recognized groups was also observed. This classification can
241 correct the GenBank annotation of deviant samples such as the BetaCoVs that grouped with AlphaCoVs,
242 and add information on the currently unclassified BetaCoVs in GenBank. This is also the first report on
243 the comprehensive classification of Nobecoviruses, of which there are currently only two recognized
244 groups: Ro-BtCoV HKU9 and Ro-BtCoV GCCDC1 (12). Our analysis of unclassified BetaCoVs suggests
245 that Nobecovirus diversity may have been underestimated in previous reports. We therefore propose the
246 subclassification of Nobecoviruses into five subgroups (2D-I to 2D-V), which can help update surveillance
247 records and facilitate monitoring of CoV populations in the wild.

248 The congruent association between the genetic clustering of bat CoVs and their bat hosts at
249 different taxonomic levels (family, subfamily, and genus) and regardless of location suggests bat taxon-
250 specific BetaCoV lineages: horseshoe and Old World leaf-nosed bats for 2B Sarbecoviruses, vesper bats
251 for 2C Merbecoviruses, fruit bats for 2D Nobecoviruses, and the closely related American leafed and
252 mustached bats for the new BetaCoV clade. Similar trends were observed upon further analysis of the
253 Nobecoviruses and their subclades: 2D-I and 2D-III BetaCoVs were found mostly in Rousettinae bats,

254 2D-II in Cynopterinae bats, and 2D-V in *Pteropus* bats. A conclusive finding could not be generated from
255 the single representative Old World leaf-nosed bat for 2E Hibecovirus. These findings support previous
256 reports (31) which we have expanded here to identify the specific bat taxon associated with each
257 BetaCoV group and a more extensive analysis of fruit bat CoVs. Network analysis showed no clear trends
258 in the geographical distribution of closely related BetaCoVs except for 2D-IV, which is composed of four
259 genetically distinct bat hosts (family Pteropodidae subfamily Pteropodinae, Epomophorinae, and
260 Rousettinae; and family Hipposideridae), but all of which are found in Africa. Hence, host specificity could
261 play a major role in BetaCoV diversity, except for 2D-IV for which geography may have a stronger
262 influence.

263 Host specificity is uncommon in other bat-infecting viruses such as Paramyxoviruses and
264 Papillomaviruses, which have been reported to have prevalent host switches (32,33). Indeed, bats have a
265 predominant viral sharing network, suggesting that cross-species transmission events are common (34).
266 In contrast, our findings support a previously proposed hypothesis that CoVs limit cross-species
267 transmission within related bat taxa (31), which is indicative of a preferred adaptation to a certain range of
268 hosts. Various lines of evidence have indicated bat-specific infectivity of CoVs. In one study, BtCoVs from
269 primary infection of *C. brachyotis* had a reduced level of replication when experimentally inoculated to
270 *Rousettus leschenaultii* (35). Similarly, the SARS-like WIV1-CoV, which was isolated from *Rhinolophus*
271 *sinicus* bats and demonstrated positive replication in *R. sinicus* cell lines, showed weak infection in
272 *Rousettus* sp. bats and cell lines (36-38). Indeed, our analysis showed that BetaCoVs from *Rousettus* sp.
273 (2D-I, 2D-III or 2D-IV) are genetically distinct from *Cynopterus* sp. (2D-II) or *Rhinolophus* sp. (2B).
274 Analysis of bat SARS-like CoV proteins demonstrated the absence of any selective pressure for evolution
275 (39), suggesting that bat CoVs have already reached optimal fitness in their host. Factors that limit
276 transmission to a different host taxon include cell surface receptors, the immune response, and
277 replication fitness (40). These factors may serve as important natural barriers in the transmission of bat
278 BetaCoVs, which can advantageously limit host jumping and co-infection, which otherwise may generate
279 new virus strains with the ability to switch animal hosts (41,42).

280 Although bat BetaCoVs are host taxon-specific, their evolutionary pathways are different from
281 bats, as has been reported in another study (31), suggesting that the virus did not have a long-term co-

282 evolution with its host. Instead, this is indicative that the currently circulating viruses may have been
283 introduced relatively recently, i.e. to the most recent common ancestors of each bat taxon but prior to
284 global dispersion and speciation, during which the virus acquired adaptation to its host. The recent
285 introduction of BetaCoVs in bats implies that other factors may have had the opportunity to influence
286 virus-host dynamics. In the succeeding discussions, we will present two deviant phenomena that
287 exemplify this: cross-taxon transmission of CoVs and bat hosts with multi-CoV lineages.

288 We provide genetic evidence for cross-taxon transmission as indicated by genetically unrelated
289 bats that host BetaCoVs of the same lineage. For example, the Mollosidae bat *Eumops glaucinus* and
290 vesper bats harbored CoVs that belong to 2C. *E. glaucinus* has a wide distribution in South America that
291 overlaps with the vesper bat *Eptesicus sp.* (43,44), which is an opportunity for cross-species transmission
292 of CoVs. Another example is the Pteropodinae bat *Dobsonia moluccensis*, *Dyacopterus spadiceus*, and
293 *M. minimus* that carried Nobecoviruses from various subgroups. *Dobsonia sp.* and Rousettinae bats are
294 primarily cave dwellers that have been documented to co-roost in the same cave habitat (45-49), which
295 could explain their shared BetaCoVs from lineage 2D-I. Evidence of forage site overlap has also been
296 documented for *Cynopterus sp.* and other Pteropodinae bats such as *M. minimus* which also feeds on
297 banana and palm trees, and *Dyacopterus spadiceus* which have been documented to feed together with
298 *Cynopterus horsfieldii* in the same foliage site (45). Here, we reported that these Cynopterinae and
299 Pteropodinae bats have BetaCoVs that commonly belong to 2D-II. A final example is 2D-IV, which is
300 composed of four genetically distinct bat hosts from Epomophorinae, Pteropodinae (*E. helvum*),
301 Rousettinae (*R. aegyptiacus*), and Hipposidridae/Old World leaf-nosed bats (*T. persicus*). The diverse
302 hosts of 2D-IV could be explained by overlaps in foraging, roosting, and distribution of African bats. For
303 example, *Rousettus aegyptiacus* has been reported to share the same foraging site with *Epomophorus*
304 *gambianus* (50). Meanwhile, *Eidolon helvum* roosts gregariously, exhibiting an annual migration pattern
305 as a function of food supply and has a wide distribution range in the African continent (51-53), which
306 overlaps with the distribution and range of other Epomophorinae fruit bats in Africa. *Triaenops persicus* is
307 also widely distributed in Eastern Africa, Middle East, and South Western Asia (54,55). Altogether, these
308 imply that cross-taxon transmission of CoVs is most likely facilitated by close interactions brought about
309 by an overlap of roosting and foraging habitat as well as geographic range of the bat hosts.

310 Our analysis also revealed that certain bat taxa such as Rousettinae, Hipposideridae (Old World
311 fruit bats), and Pteropodinae harbor multi-CoV lineages. We hypothesize that certain host factors such as
312 conserved cellular receptor motifs unique to some bat families are accessible to various forms of the CoV
313 Spike protein (S) specifically the corresponding receptor-binding motif (RBM), thereby predisposing them
314 to infection by various CoV lineages. It has been reported that different receptor-binding S1 subunit C-
315 terminal domains (S1-CTDs) from different coronavirus lineages can recognize the same receptor (56).
316 For example, the Lys353 amino acid in the angiotensin-converting enzyme 2 (ACE 2) plays a crucial role
317 for the binding of the SARS-CoV and the HCoV-NL63, both of which are very divergent coronaviruses,
318 the former being a Betacoronavirus, and the latter belonging to the alphacoronavirus lineage.
319 Independent evolution of different RBDs in coronaviruses could lead to the recognition of the same virus-
320 binding hotspot (56). Bats that can host a wide range of CoVs have the potential to propagate novel
321 viruses. It is therefore recommended that the BtCoV database be expanded through sustained
322 surveillance efforts covering more bat species especially from these three families in order to determine
323 their full range of CoV lineages.

324 The phylogenetic analysis in this study points to bats as potential origins of other mammalian
325 CoVs (2A). Switching to another taxon would require specific genetic alterations that will facilitate
326 infection of a different host species (40). This entails a strong selection pressure, and all the more when
327 switching to other animal hosts (35-38). A good demonstration of this is the evolution of human SARS-
328 CoV which is believed to have occurred in a stepwise fashion, with the spike protein undergoing early
329 selective pressure probably to mediate the switch from animal to human hosts, followed by the RdRp in
330 the late stages to facilitate a more efficient replication in humans (42). Furthermore, the human MERS-
331 CoV EMC/2012 was found to replicate in *Artibeus jamaicensis* bats, as well as in various cell lines from
332 different bat families that have never been reported to host MERS-CoV strains (37,57). Viral strains with
333 broad-spectrum tropism such as human SARS and MERS CoV are the result of an evolutionarily
334 acquired ability that combined the use of new receptors, host immune evasion, and efficient replication in
335 various host species (36,38,57,58). Anthropogenic activities such as climate changes affect the
336 distribution of previously geographically restricted disease vectors (59). Continued ecological imbalances
337 that alter bat distribution may eventually lead to loss of host specificity for bat BetaCoVs through cross-

338 taxon transmission and adaptation of multiple CoV lineages. Diverse wildlife-livestock-human interfaces
339 created by urbanization (60) could further increase the selection pressure resulting to spillover events in
340 human populations. For example, SARS-CoV likely evolved to infect humans through a series of
341 transmission events due to close or sustained contact between humans and animals in a wildlife market
342 in China (61). Furthermore, the recent SARS-CoV2 outbreak in China has been reported to originate from
343 a seafood market in Wuhan with exposure to wild animals (62). Considering all these factors, another
344 novel human CoV outbreak originating from bats is imminent. These highlight the need to monitor and
345 maintain the natural state of CoVs in the wild by strengthening routine surveillance of circulating CoVs,
346 proper urban planning to minimize the destruction of wildlife habitats, and limiting wildlife-livestock-human
347 interfaces such as by controlling wildlife consumption.

348 The detection rate of BtCoVs has been reported at a range of 2-30% in bats from various Asian
349 countries, wherein our 14.29% detection rate in Southern Philippines is within range (63-67). Moreover,
350 the 21.2% detection rate of CoVs in *Cynopterus brachyotis* from this study is lower compared to two
351 separate studies in Northern Philippines at 37% and 39%, respectively (35,63), but higher compared to
352 other Southeast Asian countries such as Thailand (11%) and Singapore (5.6%) (65,66). *C. brachyotis* is
353 locally abundant and widely distributed throughout urbanized and secondary forests in both South and
354 Southeast Asian regions, and have a high fruit species diversity in its diet (68). It is therefore
355 recommended to explore the incidence of CoVs in the wild population of *C. brachyotis* in the Philippines,
356 which may present a higher risk for future spillover infection in animal and/or human populations due to
357 their presence in urban communities. On the other hand, the absence of CoV detection in other fruit bat
358 species in this study does not rule out the possibility of these bats as reservoirs. This could have been
359 due to sampling bias, i.e. non-*Cynopterus* species comprised only ~30% of the captured bats, which
360 highlights the need to strengthen surveillance efforts for BtCoVs in individual countries to estimate the
361 true burden of viral diversity and distribution. Should there be a novel zoonotic CoV arising from fruit bats
362 such as *C. brachyotis*, it is predicted to be genetically distinct from SARS-CoV, SARS-CoV2, or MERS-
363 CoV. However, this novel virus may be just as virulent or highly contagious.

364

365 **Materials and Methods**

366 **Bat sampling and tissue collection**

367 BtCoV samples from Southern Philippines were obtained from an exploratory surveillance. Five
368 sampling sites consisting of two agricultural sites, two residential sites and one forest site were selected
369 for bat collection at Malagos, Davao City. Forty-nine individuals, all fruit bats, non-threatened and non-
370 endemic, were collected through purposive sampling for two nights on November 16 and 17, 2018 and
371 their morphometric measurements were recorded. Bat samples collected were identified using the Key to
372 the Bats of the Philippines by Ingle and Heaney (1992) (69). Bat samples were anesthetized through an
373 intraperitoneal injection of 0.1 ml tiletamine-zolazepam and euthanized via cardiac exsanguination to
374 obtain small and large intestine samples. Prior to the conduct of the study, Wildlife Gratuitous Permit
375 (WGP No. XI-2018-07) and IACUC approval (protocol no.: 2018-019) were secured from the Department
376 of Environment and Natural Resources XI and the Institutional Animal Care and Use Committee of the
377 University of the Philippines Manila, respectively.

378

379 **Coronavirus detection**

380 Genomic RNA was extracted from small intestine and large intestine samples using the SV Total
381 RNA Isolation kit (Promega, USA) according to the manufacturer's instructions. All RNA extracts were
382 subjected to reverse transcription polymerase chain reaction (RT-PCR) using the one-step RT-PCR kit
383 (Qiagen, USA) and PanCoV F2 (5'-AAR TTY TAY GGH GGY TGG-3') and PanCoV R1 (5'-GAR CAR
384 AAT TCA TGH GGD CC-3') primers (70). The RT-PCR mix was prepared as follows: 0.4 µl One-step RT-
385 PCR enzyme mix, 0.4 µl 10 mM dNTP mix, 2 µl 5x One-step RT-PCR buffer, 0.2 µl of 10 µM each of
386 PanCoV F2 and PanCoV R1 primers, 4.0 µl of RNase-Free water and 3 µl RNA extracts for a total of 10
387 µl per reaction. The cycling conditions were as follows: 30 minutes at 50°C, 15 minutes at 95°C, 40 cycles
388 at 94°C for 40 seconds, 48°C for 40 seconds and 72°C for 1 minute. The final extension was at 72°C for
389 10 minutes. Nested-PCR was subsequently performed to amplify a 435 bp bat specific region of the RNA
390 dependent RNA polymerase (RdRp) gene using in-house designed primers BatCoV F1 (5'-
391 TGACAGAGCACTGCCCAA-3') and BatCoV R1 (5'-TTGTAACAAACAACGCCATC-3') (71), and the 2X
392 Taq Master Mix (Vivantis, Subang Jaya, Malaysia). The nPCR mix was prepared as follows: 5 µl 2X Taq
393 Master Mix, 0.4 µl of 10 µM each of BatCoV F1 and BatCoV R1 primers, 2 µl one-step RT-PCR product

394 and 2.2 µl nuclease-free water for a total of 10 µl per reaction. The cycling conditions for nPCR were as
395 follows: 2 minutes at 94°C, 35 cycles of 40 seconds at 94°C, 40 seconds at 48°C, 1 minute at 72°C, and a
396 final extension for 10 minutes at 72°C. The expected 435 bp amplicon of the BtCoV RdRp gene was
397 visualized through electrophoresis using a 1.5% agarose gel.

398

399 **Sequence processing**

400 Positive amplicons with an expected size of 435 bp were excised and purified using the GF-1
401 AmbiClean Kit (Vivantis, Subang Jaya, Malaysia) and were sent to Macrogen, Korea for standard DNA
402 sequencing. Sequences were cleaned using the FinchTV software (Geospiza, USA) and distance
403 analysis was performed using the Basic Local Alignment Search Tool (BLAST) of the National Center for
404 Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>).

405

406 **Phylogenetic analysis of global CoV sequences**

407 CoV sequences obtained from this study, including 223 RdRp sequences of BtCoVs, 22 Human
408 CoV sequences which include SARS, MERS and the 2019-nCoV isolated from patients, and 19 CoV
409 sequences from other animal hosts such as camels, cats, dogs and pigs that were obtained from the
410 National Center for Biotechnology Information (NCBI) database were used for phylogenetic analysis.
411 Multiple sequence alignment was performed with the MAFFT software using the default algorithm and the
412 leave gappy regions function. The final alignment was trimmed and cleaned in MEGA 7 software in order
413 to come up with a 325 bp alignment of the CoV partial RdRp gene. The best phylogenetic model was
414 calculated using jModelTest v.2.1.10 (72). The executable xml file for phylogenetic analysis was prepared
415 using BEAUTi v.1.10.4 with the GTR+G+I DNA substitution and site heterogeneity model, which had the
416 lowest Bayesian Information Criterion (BIC) as previously calculated in jModelTest, a length of chain of
417 100 million, strict molecular clock, and coalescent constant size model. The rest of the tree priors were
418 set to the default value. Phylogenetic inference was performed using BEAST v.1.10.4 and the log files
419 were evaluated using Tracer v.1.7.1 to see if the estimated sampling size (ESS) values for most of the
420 continuous parameters are sufficient (>200) (73,74). A Maximum Clade Credibility (MCC) tree was
421 generated using TreeAnnotator v.1.10.4. and the resulting MCC tree was visualized with FigTree v.1.4.4

422 (<http://tree.bio.ed.ac.uk/software/figtree>). Results from phylogenetic analysis were compared with
423 principal coordinate analysis using the pairwise distance matrix of the CoV sequences using Past 3 (75)
424 and with the network analysis using median joining in terms of major clades, geographical region and
425 host genus.

426

427 **Phylogenetic analysis of bats**

428 *Cytochrome B* (Cyt B) gene of 43 different bat species representing the bat hosts of the
429 betacoronavirus lineage were obtained from the NCBI database. Phylogenetic analysis was performed in
430 the same manner as previously described for CoV sequences using a GTR+G+I DNA substitution and
431 heterogeneity model, which had the lowest Bayesian Information Criterion (BIC) as calculated in
432 jModelTest. The resulting bat phylogenetic trees were plotted against the CoV phylogenetic tree to
433 assess virus and host evolutionary congruence.

434

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441

442 **Conflict of Interest**

443 The authors declare no conflict of interest. The funders had no role in the design of the study; in the
444 collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish
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446

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649 Table 1. Summary of fruit bats collected from Malagos, Davao City and CoV detection.

Bat species	Collection sites	No. of samples	No. positive for CoV		Total positive for COV (%)
			small intestine	large intestine	
<i>C. brachyotis</i>	residential, agricultural, forest	33	5	2	7 (21.2)
<i>R. amplexicaudatus</i>	agricultural	10	0	0	0 (0)
<i>M. minimus</i>	residential, agricultural	5	0	0	0 (0)
<i>E. spelaea</i>	forest	1	0	0	0 (0)

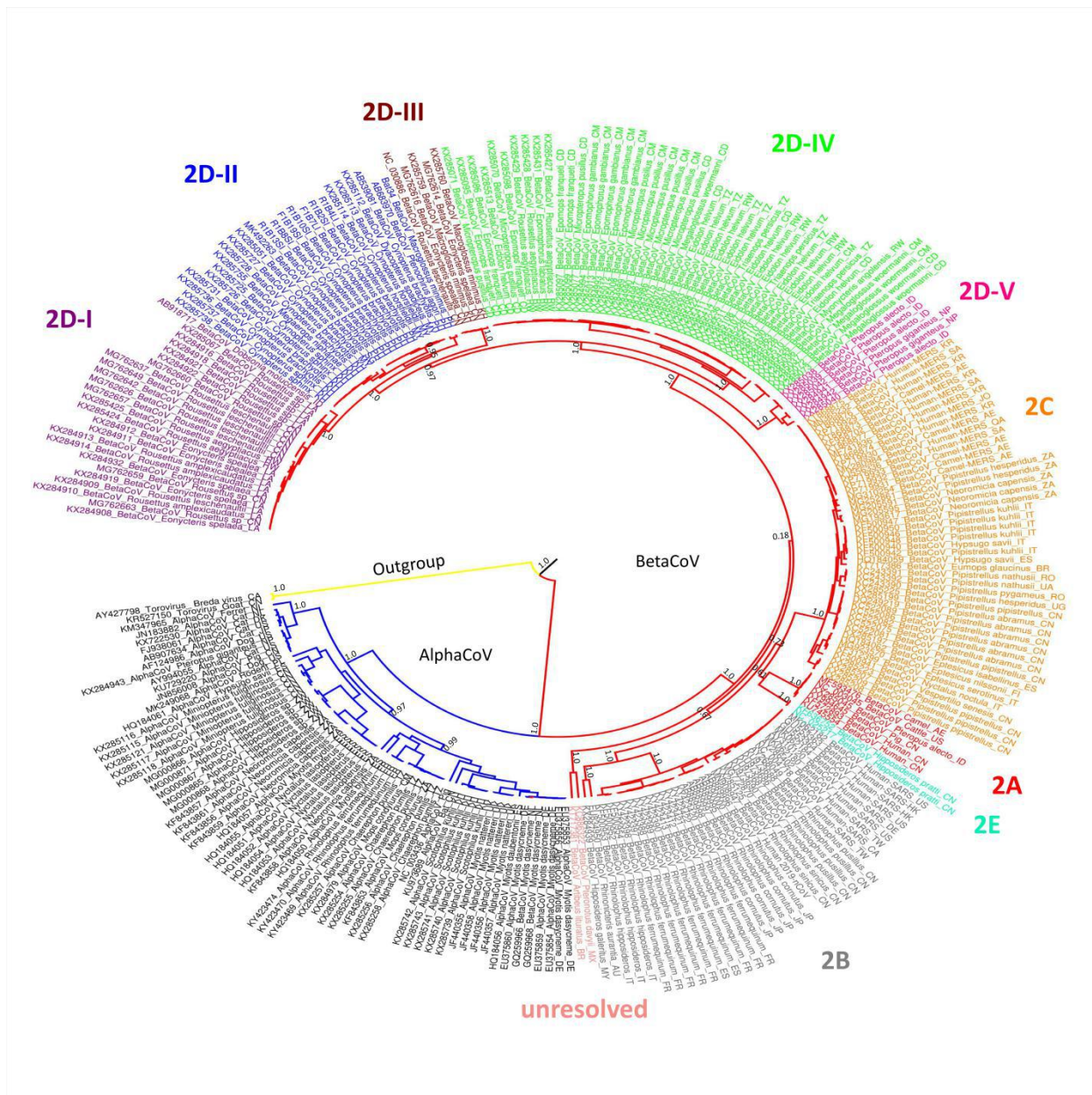
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651 Table 2. Summary of betacoronavirus groups and their corresponding regions and hosts

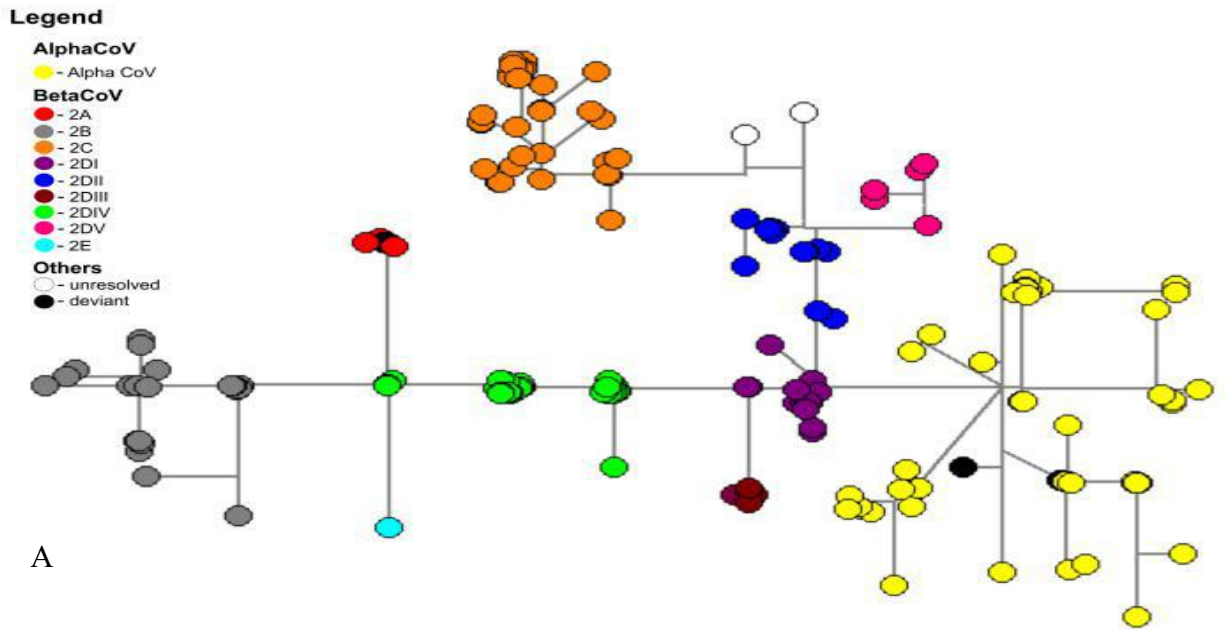
	Total	49	5	2	7 (14.3)	
	BetaCoV group	Regions	Bat hosts	Suborder¹	Family	Subfamily
	2A	SE Asia	<i>Pteropus alecto</i>	Yin	Pteropodidae	Pteropodinae
	2B	Australia	<i>Hipposideros galeritus</i>	Yin	Hipposideridae	n/a
		Europe	<i>Rhinolophus cornutus</i>	Yin	Rhinolophidae	n/a
		E Asia	<i>Rhinolophus ferrumequinum</i>		Rhinolophidae	n/a
			<i>Rhinolophus</i>	Yin		
		SE Asia	<i>hipposideros</i>		Rhinolophidae	n/a
			<i>Rhinolophus pusilus</i>	Yin	Rhinolophidae	n/a
			<i>Rhinolophus sinicus</i>	Yin	Rhinolophidae	n/a
			<i>Rhinonictes aurantia</i>	Yin	Hipposideridae	n/a
		2C	Africa	<i>Eptesicus isabellinus</i>	Yang	Vespertilionidae
	E Asia		<i>Eptesicus nilssonii</i>	Yang	Vespertilionidae	n/a
	M East		<i>Eptesicus serotinus</i>	Yang	Vespertilionidae	n/a
	S America		<i>Eumops glaucinus</i>	Yang	Molossidae	n/a
			<i>Hypsugo savii</i>	Yang	Vespertilionidae	n/a
			<i>Neoromicia capensis</i>	Yang	Vespertilionidae	n/a
			<i>Nyctalus noctula</i>	Yang	Vespertilionidae	n/a
			<i>Pippistrellus abramus</i>	Yang	Vespertilionidae	n/a
			<i>Pippistrellus hesperidus</i>		Vespertilionidae	n/a
			<i>Pippistrellus kuhlii</i>	Yang	Vespertilionidae	n/a
			<i>Pippistrellus nathusii</i>	Yang	Vespertilionidae	n/a
			<i>Pippistrellus pipistrellus</i>		Vespertilionidae	n/a
			<i>Pippistrellus pygmaeus</i>	Yang	Vespertilionidae	n/a
	<i>Vespertilio senesis</i>		Yang	Vespertilionidae	n/a	
	2D-I	Africa	<i>Dobsonia moluccensis</i>	Yin	Pteropodidae	Pteropodinae
		E Asia	<i>Eonycteris spelaea</i>	Yin	Pteropodidae	Rousettinae
			<i>Rousettus</i>	Yin		
		SE Asia	<i>amplexicaudatus</i>		Pteropodidae	Rousettinae
			<i>Rousettus leschenautii</i>	Yin	Pteropodidae	Rousettinae
		<i>Rousettus sp.</i>	Yin	Pteropodidae	Rousettinae	
	2D-II	SE Asia	<i>Cynopterus brachyotis</i>	Yin	Pteropodidae	Cynopterinae
			<i>Cynopterus horsefieldii</i>	Yin	Pteropodidae	Cynopterinae
			<i>Cynopterus sphinx</i>	Yin	Pteropodidae	Cynopterinae
			<i>Dyacopterus spadiceus</i>		Pteropodidae	Pteropodinae
			<i>Macroglossus minimus</i>	Yin	Pteropodidae	Macroglossini
			<i>Ptenochirus jagori</i>	Yin	Pteropodidae	Cynopterinae

2D-III	E Asia	<i>Eonycteris spelaea</i>	Yin	Pteropodidae	Rousettinae
	SE Asia	<i>Macroglossus minimus</i>	Yin	Pteropodidae	Macroglossini
		<i>Rousettus leschenautii</i>	Yin	Pteropodidae	Rousettinae
2D-IV	Africa	<i>Eidolon helvum</i>	Yin	Pteropodidae	Pteropodinae
		<i>Epomophorus gambianus</i>	Yin	Pteropodidae	Epomophorinae
		<i>Epomophorus labiatus</i>	Yin	Pteropodidae	Epomophorinae
		<i>Epomops franqueti</i>	Yin	Pteropodidae	Epomophorinae
		<i>Megaloglossus woermanii</i>	Yin	Pteropodidae	Epomophorinae
		<i>Micropterus pusillus</i>	Yin	Pteropodidae	Epomophorinae
		<i>Myonycteris angolensis</i>	Yin	Pteropodidae	Epomophorinae
		<i>Rousettus aegyptiacus</i>	Yin	Pteropodidae	Rousettinae
		<i>Triaenops persicus</i>	Yin	Hipposideridae	n/a
		2D-V	S Asia	<i>Pteropus alecto</i>	Yin
SE Asia	<i>Pteropus giganteus</i>		Yin	Pteropodidae	Pteropodinae
2E	E Asia	<i>Hipposideros pratti</i>	Yin	Hipposideridae	n/a
unresolved	S America	<i>Artibeus lituratus</i>	Yang	Phyllostomidae	n/a
		<i>Pteronotus davyi</i>	Yang	Mormoopidae	n/a

652 ¹Yin= Yinpterochiroptera, Yang= Yangochiroptera

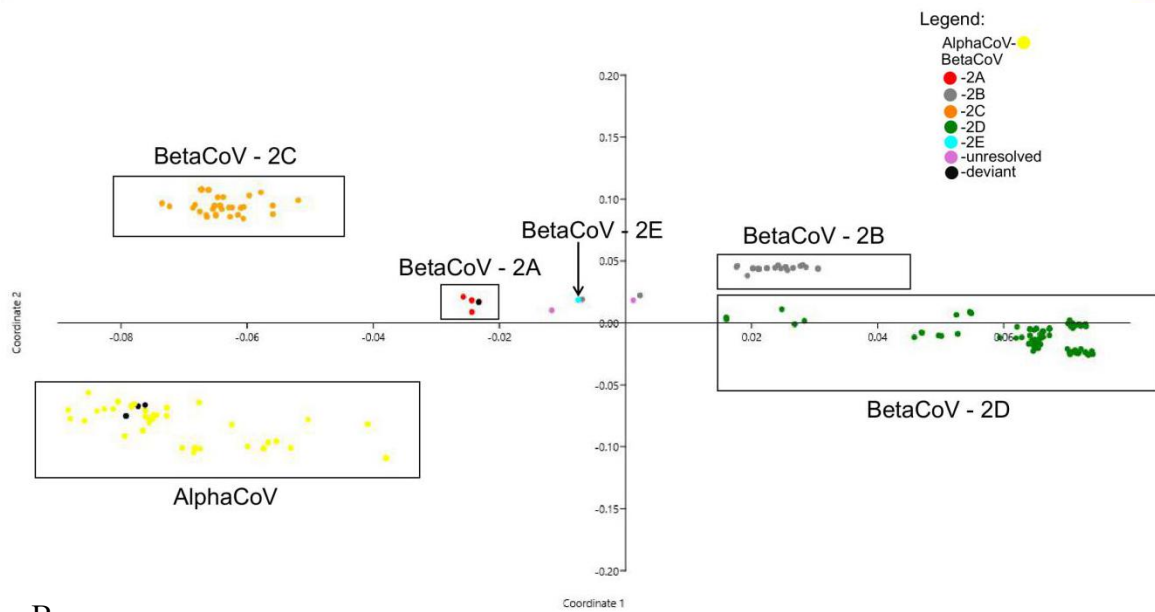


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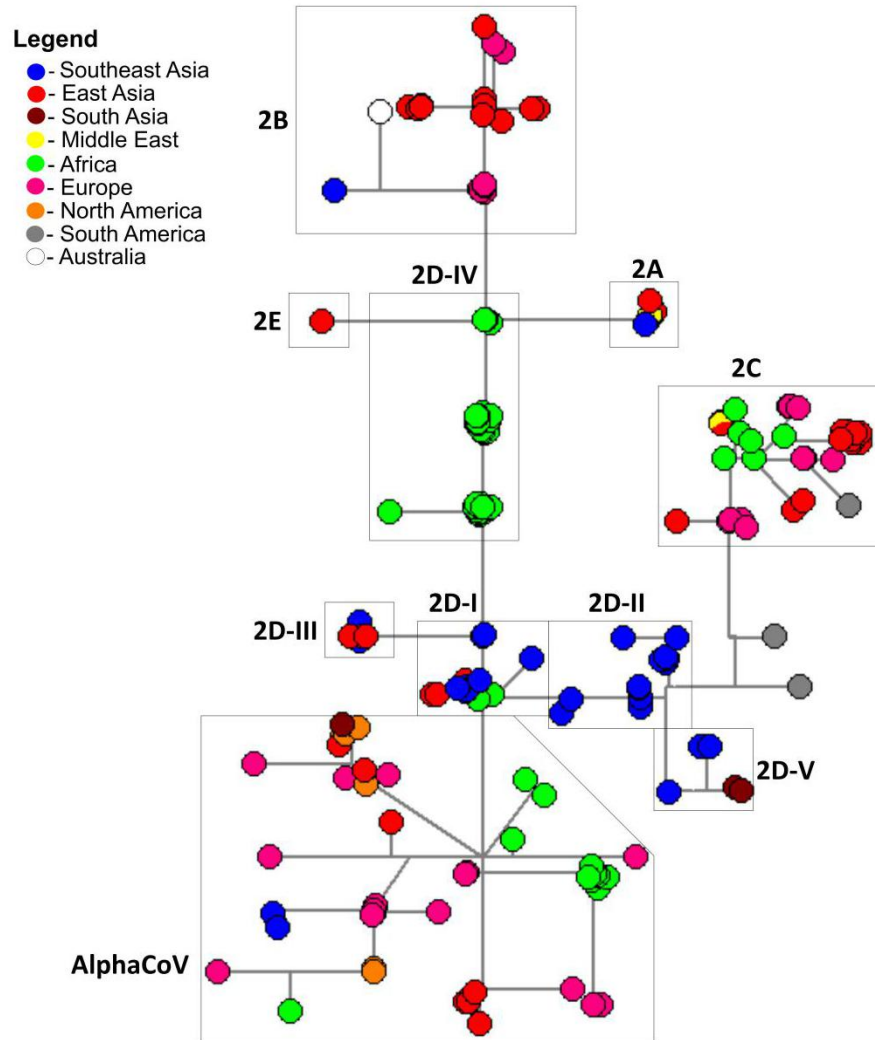
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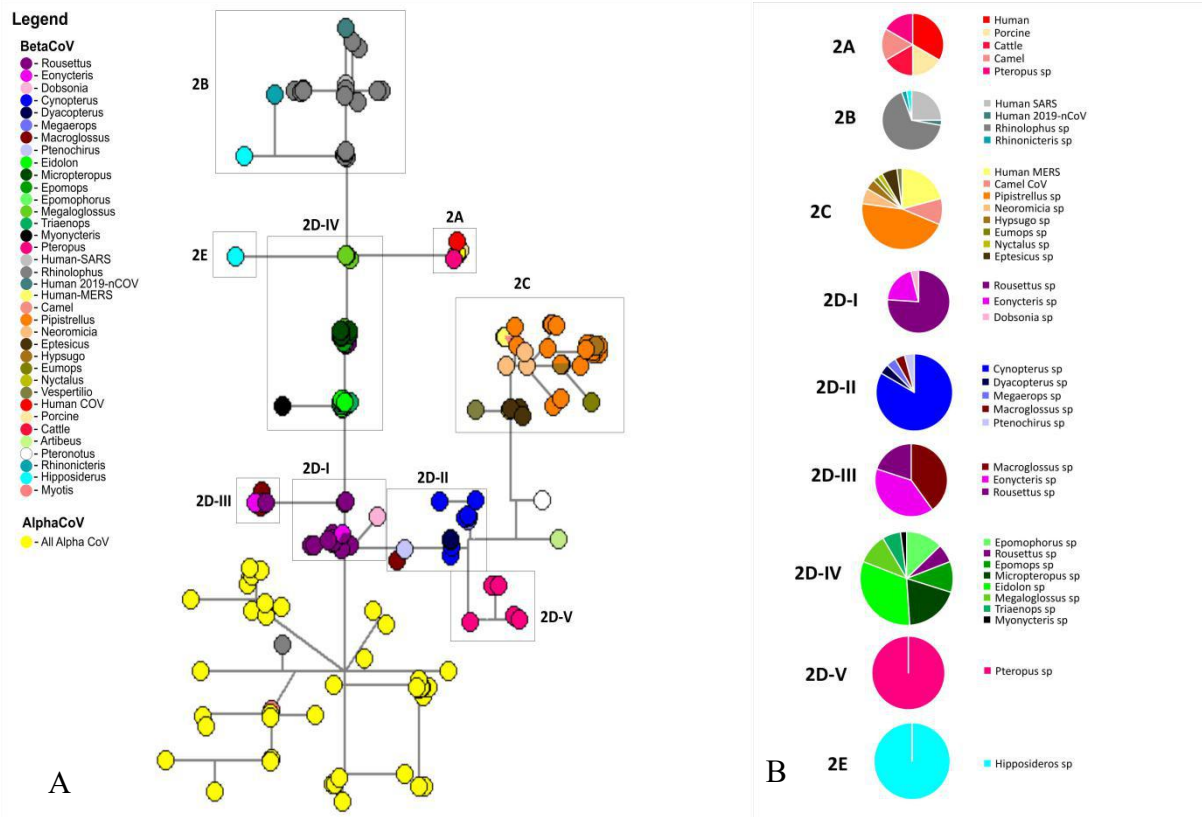
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Figure 2. Genetic clustering of global CoVs using, a) network analysis through median joining of RdRp gene sequences, and b) principal coordinate analysis of the distance matrix of the RdRp gene sequences.

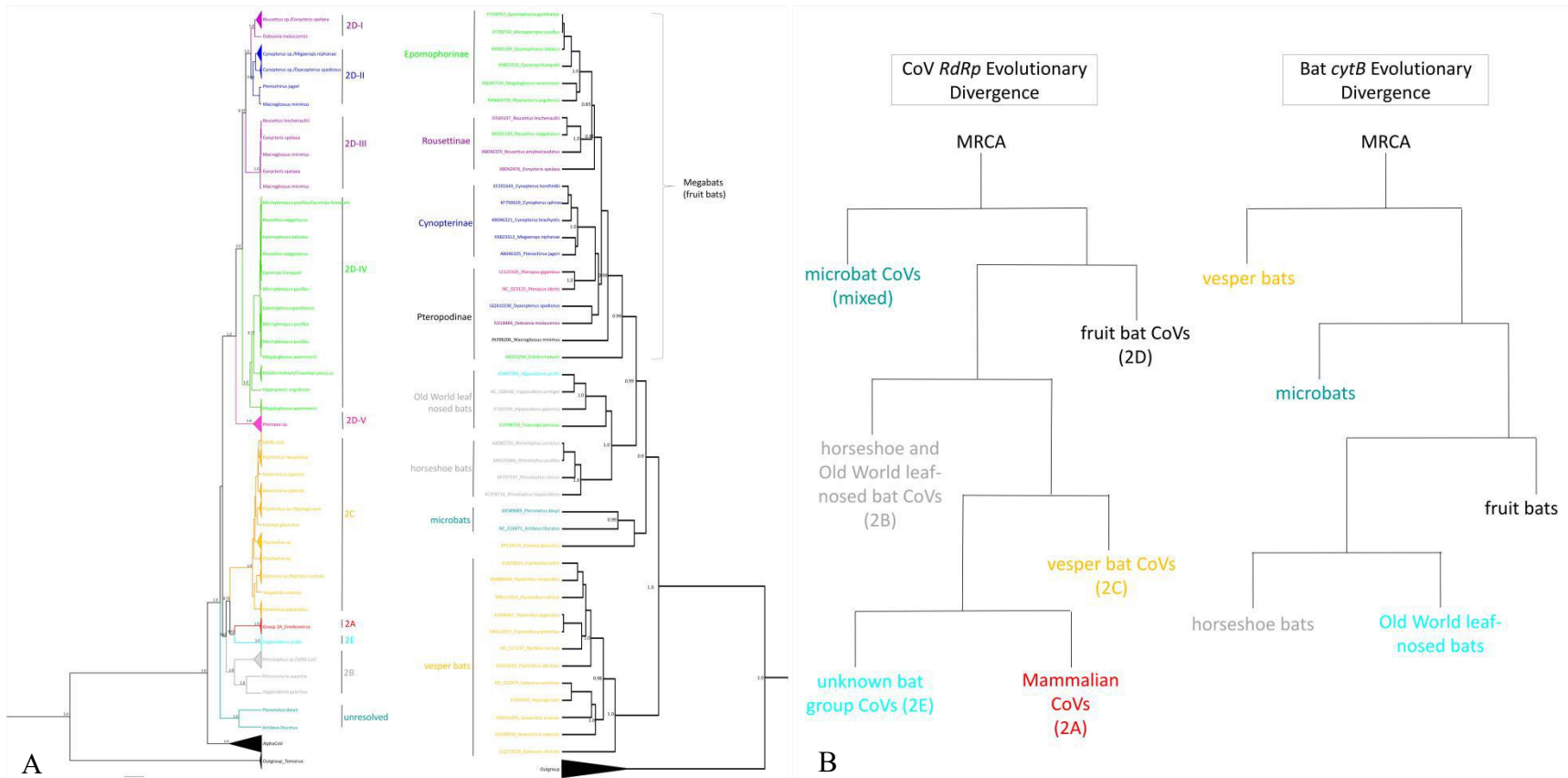


666
667 Figure 3. Regional distribution of CoVs from bats, domestic and wild animals, as well as humans using
668 network analysis of RdRp gene sequences.
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Figure 4. Host composition of betacoronavirus lineages as illustrated in the (a) network analysis of RdRp gene sequences, and (b) pie chart distribution of hosts per clade or subclade.



673
 674 Figure 5. Phylogenetic relationships between CoVs (*RdRp* gene) and their bat host (*Cyt B* gene), A) bayesian phylogenetic trees generated using
 675 bayesian inference (BEAST v.1.10.4) with the GTR+G+I DNA substitution and site heterogeneity model, strict molecular clock and coalescent
 676 constant size, with posterior values of the well supported clades written in the nodes, B) evolutionary divergence patterns of CoVs and their bat
 677 hosts based from the generated Bayesian phylogenetic trees.