Understanding the Divergent Evolution and Epidemiology of H3N8 Influenza Viruses in Dogs and Horses

Brian R. Wasik[1], Evin Rothschild[1], Ian E.H. Voorhees[1], Stephanie E. Reedy[2], Pablo R. Murcia[3], Nicola Pusterla[4], Thomas M. Chambers[2], Laura B. Goodman[5], Edward C. Holmes[6], James C. Kile[7], Colin R. Parrish[1,8]

1) Baker Institute for Animal Health, Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA.
2) Department of Veterinary Science, Gluck Equine Research Center, University of Kentucky, Lexington, KY 40546, USA.
3) MRC-University of Glasgow Centre for Virus Research, School of Infection and Immunity, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, Scotland, UK.
4) Department of Medicine & Epidemiology, School Veterinary Medicine, University of California, Davis, CA 95616, USA.
5) Baker Institute for Animal Health, Department of Public and Ecosystems Health, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA.
6) Sydney Institute for Infectious Diseases, School of Medical Sciences, University of Sydney, Sydney, New South Wales, Australia.
7) Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30329, USA.
8) Corresponding author. Colin Parrish, crp3@cornell.edu, 607-379-4233

ABSTRACT

Cross-species virus transmission events can lead to dire public health emergencies in the form of epidemics and pandemics. One example in animals is the emergence of the H3N8 equine influenza virus (EIV), first isolated in 1963 in Miami, Florida, USA, after emerging among horses in South America. In the early 21st century the American lineage of EIV diverged into two 'Florida' clades that persist today, while an EIV transferred to dogs around 1999 and gave rise to the H3N8 canine influenza virus (CIV), first reported in 2004. Here, we compare CIV in dogs and EIV in horses to reveal their host-specific evolution, to determine the sources and connections between significant outbreaks, and to gain insight into the factors controlling their different evolutionary fates. H3N8 CIV only circulated in North America, was geographically restricted after the first few years, and went extinct in 2016. Of the two EIV Florida clades, clade 1 circulates widely and shows frequent transfers between the USA and South America, Europe and elsewhere, while clade 2 was globally distributed early after it emerged, but since about 2018 has only been detected in Central Asia. Any potential zoonotic threat of these viruses to humans can only be determined with an understanding of its natural history and evolution. Our comparative analysis of these three viral lineages reveals distinct patterns and rates of sequence variation yet with similar overall evolution between clades, suggesting epidemiological intervention strategies for possible eradication of H3N8 EIV. (242 words)

IMPORTANCE

The emergence of viruses in new hosts is a threat to human and animal health. The H3N8 equine influenza virus (EIV) emerged in 1963 by transfer of an avian influenza virus, and the H3N8 canine influenza virus (CIV) subsequently emerged in 1999 when EIV transferred to dogs. H3N8 CIV persistently circulated in only a few locations in the USA, and has not been detected since 2016. In the same period H3N8 EIV has circulated as two separate clades, one in North America and other regions of the world, while the other currently appears to be found only in

Central Asia. By comparing the hosts, epidemiology, and evolution of these influenza viruses we explain how these lineages had different evolutionary fates, and show why elucidating these evolutionary processes is key to understanding zoonotic disease and viral emergence. (137 words)

Introduction

The processes driving virus outbreak epidemiology and cross-species virus transmission are topics of great importance. Studying viruses involved in emerging outbreaks in animals can reveal the fundamental processes that mediate successful transfer between hosts and the subsequent establishment of viral variants as epizootic or even zoonotic and pandemic pathogens. Such studies also suggest approaches to protecting humans and other animals from potential zoonotic viruses by preventing cross-species epizootic outbreaks, as well as new methods for blocking the emergence of transferred viruses or reducing the adaptation of viruses to new hosts, thereby reducing their pandemic potential (1–4).

Most subtypes of influenza A virus (IAV) are well established in their reservoir hosts, mainly birds living in aquatic environments (5). However, several subtypes appear to have a relatively high propensity for infecting other animal hosts, including domestic poultry, humans, swine, ferrets, seals, horses, and most recently dogs (5, 6). Some mammals occasionally suffer outbreaks from avian origin influenza, including seals and mink notably during recent spread of highly pathogenic avian influenza (HPAI) H5N1, that may suggest new subtype risks (7, 8). Most spillovers cause short-term outbreaks that rapidly die out, while some have transitioned from emergent to epidemic or pandemic pathogens in their mammalian hosts. In human populations, some IAV subtypes (primarily H1N1 and H3N2 in the modern era) have become endemic diseases (9). IAV are members of the family *Orthyomyxoviridae*, and have a negative-sense RNA genome arranged as eight segments: PB2, PB1, PA, HA, NP, NA, M, and NS (9).

Horses have historically been an important mammalian host for IAV, likely including epidemics in the 1870s in North America that posed a significant burden on animal health, as well as on a human society that was highly dependent on horses (10). In the 20th century, horses were infected by an H7N7 virus that was first recognized in 1956 and became extinct in the late 1970s (11). H3N8 equine influenza virus (EIV) was first isolated in 1963 in Florida, likely after being introduced along with infected horses from South America, and descendants of that

81 virus continue to circulate in horses today (12–15). The appearance and circulation of
82 reassortant H7N7 and H3N8 EIVs in the 1970s suggests that the H3N8 contributed to
83 displacement and extinction of the H7N7 subtype. Vaccines against H3N8 EIV were first
84 introduced in the late 1960s, and the period of the 1970s and early 1980s resulted in several
85 subclade divergences and increasing H3N8 diversity (13, 16). Around the year 2000 the H3N8
86 EIV population divided into different lineages: the Florida Clade 1 (FC1) was primarily in North
87 America but also observed in outbreak in other regions of the world, while the Florida Clade 2
88 (FC2) was found primarily in Europe, yet both virus clades have spread to many different
89 countries and continents (17–22). However, these clades appear to have kept some degree of
90 geographic separation, and they do not appear to have been in direct competition (23).
91 EIV is a common respiratory tract infection in horses and closely related equids, causing
92 influenza-like signs similar to those seen in humans (24). Current estimations of EIV incidence
93 in US horses comes from voluntary surveillance, and are frequently under 10% with seasonal
94 variability (25, 26). Vaccines for EIV have been available since the 1970s and include antigens
95 from the most commonly circulating strains, and those are widely used in domesticated horses
96 (27). While human influenza vaccines are updated frequently with the most widespread
97 antigenic variants, EIV vaccines are updated less often. In the United States, roughly 60% of
98 equine operations vaccinate for EIV (66.4% in 1998, 54.1% in 2005, 60.4% in 2015) (28). It is
99 unclear to what degree low vaccine effectiveness or antigenic mismatch have contributed to
100 recently observed ‘breakthrough’ outbreaks in multiple countries (22, 29–32).
101 Recommendations for vaccine formulations are regularly proposed by the World Organization
102 for Animal Health (formerly Office International des Epizooties, OIE) expert surveillance panel
103 (33, 34), but strain inclusion varies in different vaccine formulations (35). The potential for
104 horses to be sources of zoonotic disease given their close association with humans makes EIV
105 an identified neglected disease threat of concern (36, 37). Experimental inoculation of human
106 volunteers in the 1960s showed that the virus was able to replicate in humans with sufficient

107 exposure (38, 39). Only through proper surveillance and sequencing of circulating EIV in
108 different populations will a clearer picture of the virus variation be obtained. In particular, this
109 would enable targeted antigenic testing to justify the production of vaccines with a better
110 antigenic match and greater protective efficacy (34, 40). Determining the relationships between
111 viruses causing different outbreaks through surveillance will also reveal epidemiological
112 pathways that arise during epidemics, allowing better strategies for control and potentially
113 eradication.

114 A single H3N8 EIV transferred to dogs around 1999 to create the first known epidemic of
115 canine influenza virus (CIV). Molecular and epidemiological evidence suggests the epidemic
116 originated from a single, cross-species transmission event of a non-reassortant H3N8 EIV, with
117 cases being initially identified in greyhounds in a training facility in Florida in 2003 and 2004
118 (41–44). The H3N8 CIV spread to several regions of the USA around this period, likely due to
119 transport of infected greyhounds, although the virus only achieved prolonged transmission in
120 dogs within larger dense populations, including animal shelters, kennels, and canine day cares
121 (45). The virus appears to have persisted in large metropolitan areas (e.g. Denver and Colorado
122 Springs, New York City, and Philadelphia) with large shelter dog populations that had high
123 standing populations and high rates of turnover (46). While the emergence of CIV H3N8 led to
124 an epidemic, other observed spillovers of EIV H3N8 into dogs (in 2002 in the UK and 2007 in
125 Australia) did not result in onward canine transmission (47, 48). No human infections with H3N8
126 CIV were observed, despite frequent interactions (49). A second CIV arose around 2005 in
127 Asia, and that H3N2 strain appears to have been derived directly from an avian influenza virus
128 that transferred to dogs (50). H3N2 CIV appears to have circulated widely in Korea and China
129 for a number of years, and has been repeatedly introduced into the USA and Canada where it
130 has mostly caused local and self-limiting outbreaks (51). Risk assessment of H3N2 CIV
131 transmission to humans was found to be minimal (52).

132 Host transfers underpin many infectious disease epidemics or pandemics, and in recent
133 years such events have given rise to new emerging viruses in humans and other animals,
134 including the 2009 pandemic swine influenza (pdmH1N1), Ebolavirus, SARS-CoV-1 and -2, and
135 the H3N8 and H3N2 CIV (1, 53). Influenza surveillance networks (such as the World Health
136 Organization Global Influenza Surveillance & Response System, WHO GISRS) have made
137 considerable leaps in the past decade, with rapid identification of human cases of highly
138 pathogenic avian influenza (54, 55). Despite this, gaps remain in our ability to predict changes in
139 host-range that result in virus emergence, or to create early warning systems to detect and
140 mitigate outbreaks before they get underway (4, 56, 57). Strategies for controlling emerging
141 viruses primarily involve aggressively managing outbreaks after they first occur. Yet, this
142 requires a clear understanding of the routes of transmission, ongoing circulation, and likely host
143 adaptation leading to increased spread or immune evasion, and such knowledge is often lacking
144 in the early stages of outbreaks (3). It is also often unclear whether and how virus evolution in
145 the original host facilitates the initial successful spillover infection, or how the viruses later
146 evolve and adapt to their new hosts compared to evolution that occurs in their original hosts.
147 Understanding these processes in more detail may allow us to anticipate and forestall the
148 emergence of other viruses in the future.
149 As well as local outbreaks, a defining feature of the most threatening virus emergence
150 events is longer distance and international transfers that allow viral outbreaks to sustain over
151 time and develop into pandemics, particularly within human host populations (58, 59). Long-
152 distance transfers are also a feature of H3N8 EIV epidemiology, likely associated with the
153 movement of infected racing and sport horses (31, 60, 61). It is estimated that just 0.7% of
154 operations transport horses outside North America, yet these international transfers of horses
155 have made EIV a global pathogen of concern (28, 34, 62). Among dogs, H3N2 CIV has similarly
156 transferred between continents (51). In contrast, the H3N8 CIV outbreaks were more confined,
157 circulating within and between animal shelters and kennels within the central and eastern

158 regions in the United States (45, 46). Notably, that virus lineage did not appear to spread
159 outside the USA.

160 Modern genomic epidemiology has allowed the detailed examination of viral outbreaks,
161 including the spread and replacement of viral variants (2, 63–65). For example, the emergence
162 of more transmissible or antigenic variant viruses that displace earlier lineages is commonly
163 seen in evolving virus populations, including 2009 pdmH1N1, SARS-CoV-2 (B.1.1.7 'Alpha', or
164 B.1.1.529 'Omicron'), and the canine parvovirus type-2a variant that emerged and efficiently
165 replaced the earlier strains of that virus (66–72). These events highlight the importance of
166 continued surveillance and analysis of viruses; both in new hosts and in their original hosts, as
167 such comparative analysis allows us to identify mechanisms of true adaptive evolution following
168 emergence.

169 To resolve the evolution of H3N8 in dogs and horses we collected genomic data from
170 our own sequencing and public repositories of H3N8 beginning around 2001. Complete genome
171 sequences of all segments enable phylogenetic relationships and host-specific evolution to be
172 determined. We further employed use of the much larger collection of HA1 sequences (HA1
173 being the hemagglutinin 'head' containing the receptor binding domain and dominant antigenic
174 epitopes, H3 numbering 1-329). Here, we investigate the emergence the H3N8 canine influenza
175 virus (CIV) and its origin from the H3N8 equine influenza virus (EIV), describe the later
176 extinction of H3N8 in dogs while also following the continuing evolution and epidemiology of EIV
177 among horses in several geographic regions of the world.

178

179 **Results**

180 **H3N8 experienced major clade divergence in the early 2000s, including the**
181 **emergence of CIV in dogs.** Despite years of spread and evolution in horses since its first
182 emergence around 1963, H3N8 EIV diversity had largely collapsed to a single North American
183 clade by the end of the 20th century (Figure 1, inset) (17, 73). This 'Florida' clade then gave rise

184 to the FC1 and FC2 subclades. A full genome phylogeny since 2001 shows the presence of pre-
185 clade divergence viruses (preC), and viruses within the monophyletic FC2, FC1, and CIV
186 groups. Divergence occurred roughly after the appearance of Kentucky/5/2002,
187 California/8560/2002, and Newmarket/5/2003 preC (Figure 1), so that the first full-genome
188 samples from the FC2, FC1 and CIV clades are Richmond/1/2007, Wisconsin/1/2003,
189 ca/Florida/242/2003, respectively. These FC2 and FC1 sequences are currently (or closely
190 match) the EIV formulations frequently manufactured and used for vaccination (35).
191 We further gathered all detailed collection dates at best accuracy (YYYY-MM-DD) to
192 review the temporal nature of the data set. A root-to-tip regression analysis of the full genome
193 data set revealed strong clock-like structure (R^2 = 0.92) and an evolutionary rate of 1.5 x 10^{-3}
194 nucleotide substitutions/site/year (Figure S1A). It was obtained a reliable root-to-tip regression
195 for the expanded HA1 data set from 2001 to present, yet with rooting divergence from the
196 common ancestor between FC1/CIV and FC2 lineages (Figure S1B). The establishment and
197 persistence of these lineages allows a direct comparison of H3N8 influenza virus evolution in
198 novel (dog) and original (horse) hosts following the host transfer and emergence event giving
199 rise to CIV. To determine what genomic characteristics may have allowed FC1 (and FC2) to
200 persist in nature, while CIV went extinct, we analyzed the CIV, FC1 and FC2 clades since their
201 divergence in parallel (Figure 1).
202 **CIV was restricted to the United States and went extinct around 2016**. The last
203 known positive cases of H3N8 CIV were reported in the Northeast USA in 2016, two samples of
204 which (Maine and Massachusetts) were sequenced shortly thereafter by our group. No further
205 H3N8 positive cases in the USA have been reported, even though this was a time of frequent
206 canine influenza testing due to the emergence and spread of H3N2 CIV in the US in 2015 and
207 2016 (51).
208 We gathered a data set of 43 complete CIV genomes (and an expanded 88 HA1 reading
209 frame sequences) and employed the same methods as for the full H3N8 data set. Our full

210 genome CIV phylogeny (Figure 2A) closely matches the monophyletic group seen in the larger
211 H3N8 full genome data set (Figure 1) and previous analyses (74). The full genomes of CIV
212 showed a strong clock-like evolution (R^2 = 0.94) at approximately the same rate (1.8 x 10^{-3}
213 substitutions/site/year, Figure S1C) as observed with the larger H3N8 full genome data set. We
214 used the expanded HA1 data set to connect the phylogenetic tree to the geographic data
215 (Figure 2B). Subclades of CIV were highly structured by regional introduction and spread,
216 mostly localizing to outbreak centers in Florida, New York City, Philadelphia, and the cities of
217 Colorado. Secondary geographic sites appear to have involved secondary outbreaks that were
218 related by phylogenetics back to their source locations.

219 We further inferred the demographic history of these populations using Bayesian
220 analysis with a Skyline Plot (BSP) tree prior, enabling us to estimate changes in genetic
221 diversity – a measure of effective population size (N_e) – through time. For both data sets we
222 found that N_e plots correspond to virus natural history and epidemiology: large population and
223 diversity growth occurring during the initial US-wide outbreaks of 2005-2007, followed by
224 regional die out and the eventual complete extinction (Figures S2A-B).

225 **EIV FC1 is a US-centric virus that contributes to international transfers and**
226 **outbreaks**. We compiled 82 FC1 full genome and 252 HA1 sequence data sets including those
227 newly generated here. The FC1 full genome phylogeny shows a fairly linear clade divergence
228 (Figure 3). The trees show strong signatures of geographic clustering, suggesting frequent
229 single-introduction outbreaks in particular locations including South America, Europe, and other
230 regions. There is a strong clock-like evolution (R^2 = 0.99) at approximately the same rate (1.7 x
231 10^{-3} substitutions/site/year, Figure S1E) seen in the larger H3N8 full genome data set and the
232 CIV clade. We again estimated N_e changes through time, and while sampling bias and poor
233 admixture limits making broad conclusions, we were able to identify key demographic transitions
234 and data gaps (Figures S2A, S6, S7).

235 The phylogenetic tree of our expanded HA1 sequences largely paralleled the full-length
236 genome tree, further clarifying the larger geographic movements of FC1 since its divergence.
237 The overall phylogenetic structure reveals an apparent US-based continuous circulation that
238 seeds multiple international transfers and outbreaks in different regions of the world (Figure 4).
239 Some of these historical transfers have been reported and analyzed previously and confirmed
240 here (20, 75–79). Our Bayesian demographic analysis of the FC1 HA1 data set (Figure S8) also
241 shows that the effective population size largely mirrors the full-length genome data set, yet with
242 more points of transition and changes in diversity, some of which are likely revealed by the
243 increased sampling resolution (Figure S2B).

244 An analysis of recent FC1 phylogenetic structure (in both full genome and HA1 data
245 sets) reveals two closely related subclades at this point in time: the viruses circulating in the US,
246 and those collected from the 2019 European outbreak. The directions of transmission appear
247 complex, but since the virus in the United States was circulating for a few years prior to 2019,
248 that was likely transferred from the US to the United Kingdom and elsewhere in Europe. A few
249 viruses collected in California in 2019 clustered with the European subclade, noted in horses
250 that had been imported from Europe (metadata of samples collected by UC-Davis for this
251 study). Some 2020-2021 US cases in the Northeastern states of New York and Rhode Island
252 also more closely match the UK samples, suggesting likely transfers from Europe back to the
253 USA. Further sampling in the USA, particularly from the Northeast region, is needed to clarify
254 the relatedness of the North American and European FC1 viruses at this time.

255 **FC2 represents an under sampled and likely limited clade of EIV with current**
256 **circulation in some regions of Asia**. The public repositories have limited numbers of full
257 genomes from the FC2 clade, although more HA1 sequences are available. Sampling appears
258 to be relatively sparce, with multiple samples and sequences from notable outbreaks, so that
259 there is extensive genetic redundancy among the samples available. Our FC2 full genome ML
260 tree showed no variation from the full H3N8 data set analysis (Figure 5A, Figure 1). The more

261 substantial HA1 data set (n=254, Figure 5B) clearly revealed geographic distribution of recent
262 FC2 samples: while FC2 were centered in Europe between around 2000 and 2014, the clade
263 appears to have been transferred to Asia around 2009. Europe has seen fewer FC2 cases, with
264 last reports in 2017, while since 2018 only a single subclade has been present in mainland Asia.
265 The small number of full FC2 genomes (Figure S1G-H) resulted in Bayesian analyses
266 characterized by high statistical uncertainty (Figures S2A-B, S9-11).

267 We also note that FC2 isolates have been identified in various equid species (Mongolian
268 horses, Arabian horses, Donkeys), as well as dogs, although their epidemiological relevance is
269 unknown. Horses in Asia appear to be regularly exposed to avian influenza but have generally
270 failed to support a new emergent strain due to a lack of adaptation (80).

271 **H3N8 clades show distinct mutational signatures reflecting host biology and**
272 **unique epidemiology**. We next compared H3N8 evolution in different hosts to see if there were
273 obvious signatures of host-specific evolution after transfer to, and sustained transmission in,
274 dogs. The rate of genome evolution (i.e. nucleotide substitutions per site per year for each
275 segment) for each clade showed that substitution rates in CIV and FC1 were essentially the
276 same in all segments except HA (Figure 6A), suggesting that if new-host adaptation was
277 present it was masked in the general background rate of genetic drift (81, 82). In contrast, the
278 HA, NA, and M1 substitution rates are significantly greater within the FC1 clade than the FC2.
279 All H3N8 clades within horses and dogs exhibit lower substitution rates than reported for
280 influenza circulating in human host populations (huH3N2, Figure 6A), particularly in HA and NA
281 where antigenic selection would be most apparent.

282 We have previously reported comparative analyses of CIV and FC1 through 2015 (83).
283 Only two CIV isolates have been generated since this time, while FC1 has continued to circulate
284 widely in different regions of the world. Here, to better identify any residues subject to natural
285 selection, we have updated that analysis based on the many additional the full genome
286 sequences of the different clades. To do this we utilized several approaches in the Datamonkey

287 analytical toolset (https://datamonkey.org/, (84)). Using SLAC (Single-Likelihood Ancestor
288 Counting) we measured the mean d_N/d_S ratio of each segment for each clade (Figure 6B). An
289 elevated ratio is seen in the HA ORF (particularly in the HA1 antigenic domain), but with no
290 significant difference between clades. The divergent selection acting on the CIV and EIV H3N8
291 NS segment, reflected in variant segment length, has previously been analyzed in detail (85). In
292 sum, it appears that FC1 is undergoing greater evolutionary change in horses than the FC2
293 clade, or than the H3N8 CIV in dogs up until 2016.

294 **CIV early mutations suggest host adaptation to dogs, yet with no change in**
295 **epidemiology and avoidance of extinction.** The sequences of viruses spilling over into new
296 hosts will reflect the composition of the single virus that makes the initial transfer, and then will
297 likely undergo adaptation to the properties and physiology of the new host animal, including
298 optimizing onward transmission (3). We therefore compared the sequences and evolution of the
299 viruses that were closely related to the common ancestor of CIV (preC), the evolution of H3N8
300 CIV in its new host, and parallel continued evolution of the early FC1 H3N8 EIV in horses
301 (Figure 7A). This revealed multiple transitional mutations in CIV (Figure 7B), some of which
302 have been previously analyzed (42, 83, 85, 86). However, these genetic changes appear not to
303 have allowed CIV to increase its spread among the general dog populations, such that the virus
304 continued to be maintained as shelter-driven outbreaks which resulted in viral extinction, likely
305 through stochastic fade out (46). It is also likely that selection on the virus for spread within
306 dense and high turn-over shelter or kennel populations did not result in the selection of
307 mutations enabling enhanced spread in the household dog population required to develop a true
308 epidemic or pandemic (45). Despite this successful transfer of the H3N8 EIV to dogs, all other
309 spillovers have resulted in only single canine infections or short-lived local outbreaks (47).

310 It is also noteworthy that the H3N8 EIV FC1 acquired additional mutations during its
311 parallel spread in horses. There is some evidence that H3N8 may have undergone further host
312 adaptation in horses over time (87). This change in evolutionary potential of the EIV may

313 suggest that the preC consensus genome was particularly suited to clade divergence and host-
314 switching ~1999. While some antigenic selection may occur (22, 40, 88), most mutations are not
315 in the antigenic sites of the HA or NA, and likely represent genetic drift during the outbreak-
316 driven circulation of FC1 (Figure 4). Discerning true antigenic evolution from background genetic
317 drift will require greater surveillance and testing of immunity in horses.
318

319 **Discussion**

320 A detailed understanding of the replication and spread of infectious diseases allows
321 effective control strategies to be devised. While endemic human influenza outbreaks appear to
322 involve common global movement and widespread infections, IAVs in other hosts involves more
323 complex epidemiology, often with constrained geographic spread, and many outbreaks dying
324 out without obvious human interventions. The recent apparent extinction of the Influenza B
325 Yamagata lineage in humans suggest that influenza viruses may have a more tenuous foothold
326 in mammalian host population than has sometimes been assumed (89).

327 **Equine and canine H3N8 have different evolutionary fates.** The analysis presented
328 here focuses on the avian-mammalian emergent H3N8 viruses: first in their original equine
329 hosts in which they have been circulating for 60 years, and then with comparisons to the same
330 virus in dogs, to understand the divergent evolution of the same virus in different hosts. While
331 EIV continues to circulate and infect horses around the world, we report here that CIV was last
332 seen in 2016 in the Northeast United States, and is apparently now extinct.

333 H3N8 CIV was likely entirely contained to the United States during its ~17 years of
334 transmission in dogs, as no cases of this lineage have been reported in other countries. Our
335 new complete genome sequencing of the H3N8 CIV isolates from throughout the course
336 epidemic show that its geographic structure arose from an initial burst of widespread disease
337 that may have been associated with the movement of infected greyhounds. After 2007, only
338 local transmission was seen in Colorado and close by in Wyoming, up to around 2010 (90). In

339 the Northeast of the USA the virus was first reported in New York in 2005 and in Pennsylvania
340 in 2007. The virus continued to circulate in New York, where it was isolated from short-lived
341 outbreaks that occurred after introduction of virus (likely due to transport of infected dogs from
342 the shelters where the virus was endemic), and those were seen in Connecticut, Pennsylvania,
343 Vermont, Maine, and Massachusetts up until 2016 (45, 46). Our analysis confirms that the
344 outbreaks in Colorado and the Northeast were distinct, and also that after 2012 only a single
345 New York-centered lineage of virus continuing to circulate in dogs.

346 **Equine influenza virus circulates widely in the USA and in different regions of the**
347 **world.** In contrast, the H3N8 EIV has been found throughout North America for the 60 years
348 since it was first isolated. Ongoing passive surveillance systems confirm US circulation, with
349 some observed seasonality (26). However, frequent international transfers of the virus are also
350 a feature of its epidemiology. We confirm here that outbreaks in several countries around the
351 world were likely directly, or indirectly, caused by viruses from North America. Those included
352 widespread outbreaks in South America that started in 2018, and in the United Kingdom and
353 elsewhere in Europe starting in 2019. Similarly, viruses from Japan, Australia, Malaysia have
354 been closely related to viruses from the USA. Recent outbreaks in Western Africa also fit this
355 pattern, as viruses from Senegal, Nigeria, and Niger were closely related to those from South
356 America, which were due to viruses originating in the USA (76). SARS-CoV-2 drastically
357 changed human behavior during 2020 and 2021, with international travel spending being
358 reduced by 76% in 2020 compared to 2019 following international travel bans imposed by many
359 countries (91). Equine sport activities in the United States and around the world were
360 consequently impacted by varied economic curtailment, including the international transfer of
361 horses. Yet we see continued FC1 infections in the United States between 2020 and 2022, and
362 a potential inter-relatedness between US and European samples in that time, suggesting a
363 potential return to the norm. Overall, it appears that the FC1 lineage of EIV is maintained in the

364 horse population of the United States, and that provides viruses causing outbreaks in other
365 regions of the world (77).

366 **What host differences explain the different viral evolutionary fates?** As dogs
367 usually live in households as single animals or in small groups as their population structure and
368 movement patterns likely differ from that of horses. Larger numbers of susceptible dogs may be
369 found in animal shelters, kennels, or dog day cares, and these will allow increased transmission.
370 CIVs will spread rapidly within most shelters, and then the virus will die out once the population
371 has all been infected and become immune, or transmission may be stopped within the shelter
372 by quarantining affected dogs (45, 46). Horses are often found in groups or stables, yet its
373 impact on transmission is unclear. The transfer of horses for sport (including internationally) is
374 likely a large driver of EIV dissemination. Recent estimations by USDA-APHIS report that 57.8%
375 of equine operations in the US transported any resident equid by vehicle off the operation and
376 back in the preceding 12 months; 14.1% were >500 miles, 29.7% of operations transported
377 equids to an adjacent state, and 11.8% transported equids beyond adjacent states; while only
378 0.7% of operations transferred horses internationally (28). Additionally, there appear to have
379 been no international transfers of dogs infected with H3N8 CIV. Given the short lifespan of
380 dogs, limited circulation of H3N8, and the absence of a licensed vaccine until 2009, we assume
381 very little immune pressure on the CIV H3N8. Equids may be similar to humans as long-lived
382 vertebrates where relatively high levels of anti-EIV antibody immunity are induced due to either
383 infection or vaccination but wane in time between lifetime exposures (92).

384 Interestingly, the reservoir of H3N2 CIV in China and Korea appears to have been dogs
385 being bred in large numbers, with infected dogs then transported to the USA or Canada to
386 cause outbreaks among different dog populations. However, once again there has been no
387 onward transmission of that H3N2 CIV to other countries from North America, suggesting that
388 the recent US dog population structure may represent a ‘dead-end’ for endemic IAV circulation
389 (51).

390 **Conclusion.** Our analysis of H3N8 evolution in the horse and canine clades show
391 similar levels of variation that are lower than that observed for influenza in humans. The data
392 therefore suggest that host population structure and epidemiology rather than specific adaptive
393 evolution are driving the sustained transmission of FC1 in horses. Given the extinction of CIV
394 H3N8, this suggests that well targeted interventions could in turn result in extinction of EIV
395 H3N8 FC1 in horses. This likely requires a more refined epidemiological understanding of
396 circulation in the United States, updated vaccination formulation for FC1 and greater uptake. It
397 may further require screening of international transfers of horses for influenza to reduce
398 worldwide transmission, particularly the export of US-borne FC1 lineages, as well as the 'return'
399 of viruses to the USA from Europe. Rational human intervention may be necessary to 'break'
400 the host-structure epidemiology that is sustaining this virus.
401
402 **Materials and Methods**
403 **H3N8 Sample Collection.** Samples were obtained from various diagnostic centers
404 following routine passive diagnostic testing for respiratory disease (listed in Table S1, red).
405 Samples were received as either nasal or pharyngeal swab material, virus isolated in
406 culture/egg passages, or extracted total nucleic acid (tNA) first utilized for quantitative reverse
407 transcription-PCR (qRT-PCR) positivity. vRNA was extracted from clinical swab samples and
408 isolated using the QIAamp Viral RNA Mini kit. Purified vRNA or tNA was then either directly
409 used for influenza multi-segment RT-PCR or stored at -80˚C.
410 **Generation of Influenza Virus Sequences.** Genomes from samples were generated as
411 cDNAs using a whole genome multi-segment RT-PCR protocol as described previously (93,
412 94). A common set of primers (5′ to 3′, uni12a, GTTACGCGCCAGCAAAAGCAGG; uni12b,
413 GTTACGCGCCAGCGAAAGCAGG; uni13, GTTACGCGCCAGTAGAAACAAGG) that recognize
414 the terminal sequences of the influenza A segments were used in a single reaction with
415 SuperScript III OneStep RT-PCR with Platinum *Taq* DNA polymerase (Invitrogen). Following

416 confirmation by gel electrophoresis, viral cDNA was purified either by standard PCR reaction
417 desalting columns or with a 0.45X volume of AMPure XP beads (Beckman Coulter). Nextera XT
418 libraries were generated with 1 ng of cDNA material, while Nextera FLEX libraries were
419 generated with 150-200 ng of cDNA (Invitrogen). Libraries were multiplexed, pooled, sequenced
420 using Ilumina MiSeq 2 X 250 sequencing, and consensus sequences were determined for each
421 genome segment after assuring full genome coverage.

422 Consensus sequence editing was performed using Geneious Prime 2022.1.1. Paired
423 reads were trimmed using BBduk script (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-
424 guide/bbduk-guide/) and merged. Each sequence was assembled by mapping to a reference
425 sequence of a previously annotated equine or canine H3N8 sequence. Consensus positions
426 had read depth >300 and >75% identity. We did not observe unresolved positions under these
427 conditions from the samples generated in this study.

428 **Phylogenetic Analysis.** H3N8 nucleotide sequences were downloaded from the NCBI
429 Influenza Virus Database and combined with the consensus genomes generated in this study
430 (95). We focused on genomes from 2001 to present, encompassing the formation of the major
431 virus clades (FC2, FC1, CIV). Both inter- and intra-subtype reassortants were identified using
432 RDP4 (seven methods: RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan, 3seq) and
433 excluded if found to be a statistically significant outlier in two or more methods (96). Our total
434 data set comprised 157 full genomes (preC: 3, FC2: 29, FC1: 82, CIV: 43), with an expanded
435 611 HA1 reading frames (preC: 17, FC2: 254, FC1: 252, CIV: 88) (Tables S1-2). Sequences
436 were manually trimmed to their major open reading frames (PB2: 2280nt, PB1: 2274nt, PA:
437 2151nt, HA: 1695-1701nt, NP: 1497nt, NA: 1410-1413nt, M1: 759nt, and NS1: 654-693nt) and
438 either analyzed separately or concatenated with all other genome segments from the same
439 virus sample. HA1 nucleotide sequences were trimmed to cover amino acids 1-329 (H3
440 numbering). Nucleotide sequences were aligned using MUSCLE (97). Maximum likelihood (ML)
441 phylogenetic analysis was performed by PhyML (98), employing a generalized time-reversable

442 (GTR) substitution model, gamma-distributed (Γ4) rate variation among sites, and bootstrap
443 resampling. Both MUSCLE and PhyML were run under the Geneious Prime platform. ML trees
444 were visualized and annotated using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).
445 The temporal signal (i.e. molecular clock-like structure) of the data was assessed by a
446 regression of root-to-tip genetic distance against date of sampling using our ML tree and the
447 TempEst v.1.5.3 software (99). Accurate collection dating to the day (YYYY-MM-DD) was
448 utilized for all samples where this information was available. The sampling dates used for all
449 isolates are listed in Tables S1 and S2.
450 **Bayesian Phylogeny and Analysis.** We used a Bayesian Markov chain Monte Carlo
451 (MCMC) approach to better estimate virus divergence times and population dynamics. Analyses
452 were performed in BEAST v.1.10.4 (100), where MCMC sampling was performed with a strict
453 clock, a generalized time-reversable (GTR) substitution model with gamma distribution in four
454 categories (Γ4), and assuming a Bayesian Skyline Plot (BSP) prior demographic model.
455 Analyses were performed for a minimum of 100 million runs, with duplicates combined in Log
456 Combiner v1.10.4. All output results were examined for statistical convergence in Tracer v1.7.2
457 (effective sample size [ESS] ≥200, consistent traces, removed burn-in at 10-15%) (101).
458 Maximum clade credibility (MCC) phylogenetic trees were generated by Tree Annotator v1.10.4,
459 and visualized by FigTree v1.4.4.
460 **Analysis of Selection Pressures.** The relative numbers of synonymous (d_S) and
461 nonsynonymous (d_N) nucleotide substitutions per site in each ORF of each segment were
462 analyzed for the signature of positive selection (adaptive evolution, $p<0.05$ or >0.95 posterior)
463 using a variety of method packages available in the Datamonkey package
464 (https://datamonkey.org/, (84)): BUSTED (**B**ranch-site **U**nrestricted **S**tatistical **T**est
465 for **E**pisodic **D**iversification), FEL (**F**ixed **E**ffects **L**ikelihood), FUBAR
466 (**F**ast, **U**nconstrained **B**ayesian **A**pp**R**oximation), MEME (**M**ixed **E**ffects **M**odel of **E**volution), and
467 SLAC (**S**ingle-**L**ikelihood **A**ncestor **C**ounting).

468 **Graphing and Statistical Analysis.** All figure graphs were generated in GraphPad
469 Prism v.9.5.0.
470 **Data Availability.** Virus genome consensus sequences have been submitted to
471 GenBank, and accession numbers of the sequences used (and generated) for this study are
472 provided in Tables S1 and S2.
473

474 **Acknowledgements**

475 We thank the sources of the new samples used in this study, including diagnostic labs
476 and individual private veterinarians. We wish to thank members of the Parrish Lab for critical
477 feedback and support on this manuscript, particularly Wendy Weichert. SER and TMC were
478 supported by a project of the Kentucky Agricultural Experiment Station, Project No. KY-014067.
479 PRM was supported by the Medical Research Council of the United Kingdom (Grant
480 MC_UU_12014/9), the Horserace Betting Levy Board (Grants 797), the Biotechnology and
481 Biological Sciences Research Council (Grants BB/V002821/1 and BB/V004697/1). CRP (and by
482 extension BRW, ER, and IEHV) were supported by National Institutes of Health grant R01-
483 GM080533. This work was partially funded by the Influenza Division of the Centers for Disease
484 Control, Animal-Human Interface program, under contract #75D30121P12812 to CRP and LBG.
485

486 **References**

487 1. Parrish CR, Holmes EC, Morens DM, Park E-C, Burke DS, Calisher CH, Laughlin CA, Saif LJ,
488 Daszak P. 2008. Cross-species virus transmission and the emergence of new epidemic
489 diseases. Microbiol Mol Biol Rev 72:457–470.

490 2. Antia R, Regoes RR, Koella JC, Bergstrom CT. 2003. The role of evolution in the emergence
491 of infectious diseases. Nature 426:658–661.

492 3. Wasik BR, de Wit E, Munster V, Lloyd-Smith JO, Martinez-Sobrido L, Parrish CR. 2019.
493 Onward transmission of viruses: how do viruses emerge to cause epidemics after spillover?
494 Philos Trans R Soc Lond B Biol Sci 374:20190017.

495 4. Plowright RK, Parrish CR, McCallum H, Hudson PJ, Ko AI, Graham AL, Lloyd-Smith JO. 2017.
496 Pathways to zoonotic spillover. Nat Rev Microbiol 15:502–510.

497 5. Yoon S-W, Webby RJ, Webster RG. 2014. Evolution and ecology of influenza A viruses. Curr
498 Top Microbiol Immunol 385:359–375.

499 6. Parrish CR, Murcia PR, Holmes EC. 2015. Influenza Virus Reservoirs and Intermediate Hosts:
500 Dogs, Horses, and New Possibilities for Influenza Virus Exposure of Humans. Journal of
501 Virology 89:2990–2994.

502 7. Agüero M, Monne I, Sánchez A, Zecchin B, Fusaro A, Ruano MJ, del Valle Arrojo M,
503 Fernández-Antonio R, Souto AM, Tordable P, Cañás J, Bonfante F, Giussani E, Terregino C,
504 Orejas JJ. 2023. Highly pathogenic avian influenza A(H5N1) virus infection in farmed minks,
505 Spain, October 2022. Eurosurveillance 28.

506 8. Gamarra-Toledo V, Plaza PI, Inga G, Gutiérrez R, García-Tello O, Valdivia-Ramírez L,
507 Huamán-Mendoza D, Nieto-Navarrete JC, Ventura S, Lambertucci SA. 2023. First Mass
508 Mortality of Marine Mammals Caused by Highly Pathogenic Influenza Virus (H5N1) in South
509 America https://doi.org/10.1101/2023.02.08.527769.

510 9. Krammer F, Smith GJD, Fouchier RAM, Peiris M, Kedzierska K, Doherty PC, Palese P, Shaw
511 ML, Treanor J, Webster RG, García-Sastre A. 2018. Influenza. Nat Rev Dis Primers 4:3.

512 10. Morens DM, Taubenberger JK. 2010. An avian outbreak associated with panzootic equine
513 influenza in 1872: an early example of highly pathogenic avian influenza? Influenza Other
514 Respir Viruses 4:373–377.

515 11. Webster RG. 1993. Are equine 1 influenza viruses still present in horses? Equine Vet J
516 25:537–538.

517 12. Chambers TM. 2022. Equine Influenza. Cold Spring Harb Perspect Med 12:a038331.

518 13. Murcia PR, Wood JLN, Holmes EC. 2011. Genome-scale evolution and phylodynamics of
519 equine H3N8 influenza A virus. J Virol 85:5312–5322.

520 14. Waddell GH, Teigland MB, Sigel MM. 1963. A NEW INFLUENZA VIRUS ASSOCIATED WITH
521 EQUINE RESPIRATORY DISEASE. J Am Vet Med Assoc 143:587–590.

522 15. Scholtens RG, Steele JH, Dowdle WR, Yarbrough WB, Robinson RQ. 1964. U.S. EPIZOOTIC OF
523 EQUINE INFLUENZA, 1963. Public Health Rep 79:393–402.

524 16. Murcia PR, Baillie GJ, Stack JC, Jervis C, Elton D, Mumford JA, Daly J, Kellam P, Grenfell BT,
525 Holmes EC, Wood JLN. 2013. Evolution of equine influenza virus in vaccinated horses. J Virol
526 87:4768–4771.

527 17. Daly JM, Lai AC, Binns MM, Chambers TM, Barrandeguy M, Mumford JA. 1996. Antigenic
528 and genetic evolution of equine H3N8 influenza A viruses. J Gen Virol 77 (Pt 4):661–671.

529 18. Oxburgh L, Klingeborn B. 1999. Cocirculation of two distinct lineages of equine influenza
530 virus subtype H3N8. J Clin Microbiol 37:3005–3009.

531 19. Miño S, Mojsiejczuk L, Guo W, Zhang H, Qi T, Du C, Zhang X, Wang J, Campos R, Wang X.
532 2019. Equine influenza virus in Asia: phylogeographic pattern and molecular features
533 revealed the circulation of an autochthonous lineage. J Virol 93:e00116-19.

534 20. Diallo AA, Souley MM, Issa Ibrahim A, Alassane A, Issa R, Gagara H, Yaou B, Issiakou A, Diop
535 M, Ba Diouf RO, Lo FT, Lo MM, Bakhoum T, Sylla M, Seck MT, Meseko C, Shittu I, Cullinane
536 A, Settypalli TBK, Lamien CE, Dundon WG, Cattoli G. 2021. Transboundary spread of equine
537 influenza viruses (H3N8) in West and Central Africa: Molecular characterization of identified
538 viruses during outbreaks in Niger and Senegal, in 2019. Transbound Emerg Dis 68:1253–
539 1262.

540 21. Karamendin K, Kydyrmanov A, Kasymbekov Y, Khan E, Daulbayeva K, Asanova S, Zhumatov
541 K, Seidalina A, Sayatov M, Fereidouni SR. 2014. Continuing evolution of equine influenza
542 virus in Central Asia, 2007-2012. Arch Virol 159:2321–2327.

543 22. Nemoto M, Ohta M, Yamanaka T, Kambayashi Y, Bannai H, Tsujimura K, Yamayoshi S,
544 Kawaoka Y, Cullinane A. 2021. Antigenic differences between equine influenza virus vaccine
545 strains and Florida sublineage clade 1 strains isolated in Europe in 2019. Vet J 272:105674.

546 23. Bryant NA, Rash AS, Russell CA, Ross J, Cooke A, Bowman S, MacRae S, Lewis NS, Paillot R,
547 Zanoni R, Meier H, Griffiths LA, Daly JM, Tiwari A, Chambers TM, Newton JR, Elton DM.
548 2009. Antigenic and genetic variations in European and North American equine influenza
549 virus strains (H3N8) isolated from 2006 to 2007. Vet Microbiol 138:41–52.

24. Dionísio L, Medeiros F, Pequito M, Faustino-Rocha AI. 2021. Equine influenza: a comprehensive review from etiology to treatment. Anim Health Res Rev 22:56–71.

25. Pusterla N, James K, Barnum S, Bain F, Barnett DC, Chappell D, Gaughan E, Craig B, Schneider C, Vaala W. 2022. Frequency of Detection and Prevalence Factors Associated with Common Respiratory Pathogens in Equids with Acute Onset of Fever and/or Respiratory Signs (2008-2021). Pathogens 11:759.

26. Chappell DE, Barnett DC, James K, Craig B, Bain F, Gaughan E, Schneider C, Vaala W, Barnum SM, Pusterla N. 2023. Voluntary Surveillance Program for Equine Influenza Virus in the United States during 2008-2021. Pathogens 12:192.

27. Oladunni FS, Oseni SO, Martinez-Sobrido L, Chambers TM. 2021. Equine Influenza Virus and Vaccines. Viruses 13:1657.

28. 2016. Equine 2015, “Baseline Reference of Equine Health and Management in the United States, 2015.” #718.1216. USDA–APHIS–VS–CEAH–NAHMS.

29. Castro ER, Perez R, Rodriguez S, Bassetti L, Negro R, Vidal R. 2019. Epidemiological and virological findings during an outbreak of equine influenza in Uruguay in 2018. Rev Sci Tech 38:737–749.

30. Daly JM, Megid J, Langoni H, de Nardi Júnior G, Ribeiro MG. 2021. Retrospective serological survey for influenza in horses from Brazil. Braz J Microbiol 52:461–466.

568 31. Gildea S, Garvey M, Lyons P, Lyons R, Gahan J, Walsh C, Cullinane A. 2018. Multifocal Equine
569 Influenza Outbreak with Vaccination Breakdown in Thoroughbred Racehorses. Pathogens 7.

570 32. Olguin-Perglione C, Vissani MA, Alamos F, Tordoya MS, Barrandeguy M. 2020. Multifocal
571 outbreak of equine influenza in vaccinated horses in Argentina in 2018: Epidemiological
572 aspects and molecular characterisation of the involved virus strains. Equine Vet J 52:420–
573 427.

574 33. 2020. OIE Expert Surveillance Panel on Equine Influenza Vaccine Composition, Official
575 2020-1.

576 34. 2022. Expert surveillance panel on equine influenza vaccine composition. World
577 Organization for Animal Health.

578 35. Pavulraj S, Bergmann T, Trombetta CM, Marchi S, Montomoli E, Alami SSE, Ragni-Alunni R,
579 Osterrieder N, Azab W. 2021. Immunogenicity of Calvenza-03 EIV/EHV® Vaccine in Horses:
580 Comparative In Vivo Study. Vaccines (Basel) 9:166.

581 36. Sack A, Cullinane A, Daramragchaa U, Chuluunbaatar M, Gonchigoo B, Gray GC. 2019.
582 Equine Influenza Virus—A Neglected, Reemergent Disease Threat. Emerg Infect Dis
583 25:1185–1191.

584 37. Sack A, Oladunni FS, Gonchigoo B, Chambers TM, Gray GC. 2020. Zoonotic Diseases from
585 Horses: A Systematic Review. Vector Borne Zoonotic Dis 20:484–495.

586 38. Kasel JA, Couch RB. 1969. Experimental infection in man and horses with influenza A
587 viruses. Bull World Health Organ 41:447–452.

588 39. Kasel JA, Alford RH, Knight V, Waddell GH, Sigel MM. 1965. EXPERIMENTAL INFECTION OF
589 HUMAN VOLUNTEERS WITH EQUINE INFLUENZA VIRUS. Nature 206:41–43.

590 40. Nemoto M, Reedy SE, Yano T, Suzuki K, Fukuda S, Garvey M, Kambayashi Y, Bannai H,
591 Tsujimura K, Yamanaka T, Cullinane A, Chambers TM. 2023. Antigenic comparison of H3N8
592 equine influenza viruses belonging to Florida sublineage clade 1 between vaccine strains
593 and North American strains isolated in 2021-2022. Arch Virol 168:94.

594 41. Anderson TC, Bromfield CR, Crawford PC, Dodds WJ, Gibbs EPJ, Hernandez JA. 2012.
595 Serological evidence of H3N8 canine influenza-like virus circulation in USA dogs prior to
596 2004. Vet J 191:312–316.

597 42. Collins PJ, Vachieri SG, Haire LF, Ogrodowicz RW, Martin SR, Walker PA, Xiong X, Gamblin SJ,
598 Skehel JJ. 2014. Recent evolution of equine influenza and the origin of canine influenza.
599 Proc Natl Acad Sci USA 111:11175–11180.

600 43. Crawford PC, Dubovi EJ, Castleman WL, Stephenson I, Gibbs EPJ, Chen L, Smith C, Hill RC,
601 Ferro P, Pompey J, Bright RA, Medina M-J, Johnson CM, Olsen CW, Cox NJ, Klimov AI, Katz
602 JM, Donis RO. 2005. Transmission of equine influenza virus to dogs. Science 310:482–485.

603 44. Payungporn S, Crawford PC, Kouo TS, Chen L, Pompey J, Castleman WL, Dubovi EJ, Katz JM,
604 Donis RO. 2008. Influenza A virus (H3N8) in dogs with respiratory disease, Florida. Emerging
605 Infect Dis 14:902–908.

606 45. Dalziel BD, Huang K, Geoghegan JL, Arinaminpathy N, Dubovi EJ, Grenfell BT, Ellner SP,
607 Holmes EC, Parrish CR. 2014. Contact heterogeneity, rather than transmission efficiency,
608 limits the emergence and spread of canine influenza virus. PLoS Pathog 10:e1004455.

609 46. Hayward JJ, Dubovi EJ, Scarlett JM, Janeczko S, Holmes EC, Parrish CR. 2010. Microevolution
610 of canine influenza virus in shelters and its molecular epidemiology in the United States. J
611 Virol 84:12636–12645.

612 47. Crispe E, Finlaison DS, Hurt AC, Kirkland PD. 2011. Infection of dogs with equine influenza
613 virus: evidence for transmission from horses during the Australian outbreak. Aust Vet J 89
614 Suppl 1:27–28.

615 48. Daly JM, Blunden AS, Macrae S, Miller J, Bowman SJ, Kolodziejek J, Nowotny N, Smith KC.
616 2008. Transmission of equine influenza virus to English foxhounds. Emerg Infect Dis 14:461–
617 464.

618 49. Krueger WS, Heil GL, Yoon K-J, Gray GC. 2014. No evidence for zoonotic transmission of
619 H3N8 canine influenza virus among US adults occupationally exposed to dogs. Influenza
620 Other Respir Viruses 8:99–106.

621 50. Li S, Shi Z, Jiao P, Zhang G, Zhong Z, Tian W, Long L-P, Cai Z, Zhu X, Liao M, Wan X-F. 2010.
622 Avian-origin H3N2 canine influenza A viruses in Southern China. Infect Genet Evol 10:1286–
623 1288.

624 51. Voorhees IEH, Dalziel BD, Glaser A, Dubovi EJ, Murcia PR, Newbury S, Toohey-Kurth K, Su S,
625 Kriti D, Van Bakel H, Goodman LB, Leutenegger C, Holmes EC, Parrish CR. 2018. Multiple

626 Incursions and Recurrent Epidemic Fade-Out of H3N2 Canine Influenza A Virus in the United
627 States. J Virol 92:e00323-18.

628 52. Martinez-Sobrido L, Blanco-Lobo P, Rodriguez L, Fitzgerald T, Zhang H, Nguyen P, Anderson
629 CS, Holden-Wiltse J, Bandyopadhyay S, Nogales A, DeDiego ML, Wasik BR, Miller BL, Henry
630 C, Wilson PC, Sangster MY, Treanor JJ, Topham DJ, Byrd-Leotis L, Steinhauer DA, Cummings
631 RD, Luczo JM, Tompkins SM, Sakamoto K, Jones CA, Steel J, Lowen AC, Danzy S, Tao H, Fink
632 AL, Klein SL, Wohlgemuth N, Fenstermacher KJ, El Najjar F, Pekosz A, Sauer L, Lewis MK,
633 Shaw-Saliba K, Rothman RE, Liu Z-Y, Chen K-F, Parrish CR, Voorhees IEH, Kawaoka Y,
634 Neumann G, Chiba S, Fan S, Hatta M, Kong H, Zhong G, Wang G, Uccellini MB, García-Sastre
635 A, Perez DR, Ferreri LM, Herfst S, Richard M, Fouchier R, Burke D, Pattinson D, Smith DJ,
636 Meliopoulos V, Freiden P, Livingston B, Sharp B, Cherry S, Dib JC, Yang G, Russell CJ, Barman
637 S, Webby RJ, Krauss S, Danner A, Woodard K, Peiris M, Perera R a. PM, Chan MCW,
638 Govorkova EA, Marathe BM, Pascua PNQ, Smith G, Li Y-T, Thomas PG, Schultz-Cherry S.
639 2020. Characterizing Emerging Canine H3 Influenza Viruses. PLoS Pathog 16:e1008409.

640 53. Morens DM, Daszak P, Markel H, Taubenberger JK. 2020. Pandemic COVID-19 Joins
641 History's Pandemic Legion. mBio 11:e00812-20.

642 54. Hay AJ, McCauley JW. 2018. The WHO global influenza surveillance and response system
643 (GISRS)-A future perspective. Influenza Other Respir Viruses 12:551–557.

644 55. Ziegler T, Mamahit A, Cox NJ. 2018. 65 years of influenza surveillance by a World Health
645 Organization-coordinated global network. Influenza Other Respir Viruses 12:558–565.

646 56. Fagre AC, Cohen LE, Eskew EA, Farrell M, Glennon E, Joseph MB, Frank HK, Ryan SJ, Carlson
647 CJ, Albery GF. 2022. Assessing the risk of human-to-wildlife pathogen transmission for
648 conservation and public health. Ecol Lett 25:1534–1549.

649 57. Pepin KM, Lass S, Pulliam JRC, Read AF, Lloyd-Smith JO. 2010. Identifying genetic markers of
650 adaptation for surveillance of viral host jumps. Nat Rev Microbiol 8:802–813.

651 58. Lemey P, Rambaut A, Bedford T, Faria N, Bielejec F, Baele G, Russell CA, Smith DJ, Pybus OG,
652 Brockmann D, Suchard MA. 2014. Unifying viral genetics and human transportation data to
653 predict the global transmission dynamics of human influenza H3N2. PLoS Pathog
654 10:e1003932.

655 59. Worobey M, Pekar J, Larsen BB, Nelson MI, Hill V, Joy JB, Rambaut A, Suchard MA,
656 Wertheim JO, Lemey P. 2020. The emergence of SARS-CoV-2 in Europe and North America.
657 Science 370:564–570.

658 60. Murcia PR, Wood JLN. 2011. Equine influenza virus: a jumping virus that races with
659 Thoroughbred horses and greyhounds. Vet J 189:3–4.

660 61. Gildea S, Fitzpatrick DA, Cullinane A. 2013. Epidemiological and virological investigations of
661 equine influenza outbreaks in Ireland (2010-2012). Influenza Other Respir Viruses 7 Suppl
662 4:61–72.

663 62. Cullinane A, Newton JR. 2013. Equine influenza--a global perspective. Vet Microbiol
664 167:205–214.

665 63. Rochman ND, Wolf YI, Koonin EV. 2022. Molecular adaptations during viral epidemics.
666 EMBO Rep 23:e55393.

667 64. Grubaugh ND, Ladner JT, Lemey P, Pybus OG, Rambaut A, Holmes EC, Andersen KG. 2019.
668 Tracking virus outbreaks in the twenty-first century. Nat Microbiol 4:10–19.

669 65. Hadfield J, Megill C, Bell SM, Huddleston J, Potter B, Callender C, Sagulenko P, Bedford T,
670 Neher RA. 2018. Nextstrain: real-time tracking of pathogen evolution. Bioinformatics
671 34:4121–4123.

672 66. Parrish CR, Have P, Foreyt WJ, Evermann JF, Senda M, Carmichael LE. 1988. The global
673 spread and replacement of canine parvovirus strains. J Gen Virol 69 (Pt 5):1111–1116.

674 67. Elderfield RA, Watson SJ, Godlee A, Adamson WE, Thompson CI, Dunning J, Mirian F-A,
675 Blumenkrantz D, Hussell T, and Zambon M, Openshaw P, Kellam P, Barclay WS. 2014.
676 Accumulation of human-adapting mutations during circulation of A(H1N1)pdm09 influenza
677 virus in humans in the United Kingdom. J Virol 88:13269–83.

678 68. Su YCF, Bahl J, Joseph U, Butt KM, Peck HA, Koay ESC, Oon LLE, Barr IG, Vijaykrishna D,
679 Smith GJD. 2015. Phylodynamics of H1N1/2009 influenza reveals the transition from host
680 adaptation to immune-driven selection. Nat Commun 6:7952.

681 69. Hill V, Du Plessis L, Peacock TP, Aggarwal D, Colquhoun R, Carabelli AM, Ellaby N, Gallagher
682 E, Groves N, Jackson B, McCrone JT, O'Toole Á, Price A, Sanderson T, Scher E, Southgate J,
683 Volz E, Barclay WS, Barrett JC, Chand M, Connor T, Goodfellow I, Gupta RK, Harrison EM,

684 Loman N, Myers R, Robertson DL, Pybus OG, Rambaut A. 2022. The origins and molecular
685 evolution of SARS-CoV-2 lineage B.1.1.7 in the UK. Virus Evol 8:veac080.

686 70. Viana R, Moyo S, Amoako DG, Tegally H, Scheepers C, Althaus CL, Anyaneji UJ, Bester PA,
687 Boni MF, Chand M, Choga WT, Colquhoun R, Davids M, Deforche K, Doolabh D, du Plessis L,
688 Engelbrecht S, Everatt J, Giandhari J, Giovanetti M, Hardie D, Hill V, Hsiao N-Y, Iranzadeh A,
689 Ismail A, Joseph C, Joseph R, Koopile L, Kosakovsky Pond SL, Kraemer MUG, Kuate-Lere L,
690 Laguda-Akingba O, Lesetedi-Mafoko O, Lessells RJ, Lockman S, Lucaci AG, Maharaj A,
691 Mahlangu B, Maponga T, Mahlakwane K, Makatini Z, Marais G, Maruapula D, Masupu K,
692 Matshaba M, Mayaphi S, Mbhele N, Mbulawa MB, Mendes A, Mlisana K, Mnguni A, Mohale
693 T, Moir M, Moruisi K, Mosepele M, Motsatsi G, Motswaledi MS, Mphoyakgosi T, Msomi N,
694 Mwangi PN, Naidoo Y, Ntuli N, Nyaga M, Olubayo L, Pillay S, Radibe B, Ramphal Y, Ramphal
695 U, San JE, Scott L, Shapiro R, Singh L, Smith-Lawrence P, Stevens W, Strydom A, Subramoney
696 K, Tebeila N, Tshiabuila D, Tsui J, van Wyk S, Weaver S, Wibmer CK, Wilkinson E, Wolter N,
697 Zarebski AE, Zuze B, Goedhals D, Preiser W, Treurnicht F, Venter M, Williamson C, Pybus
698 OG, Bhiman J, Glass A, Martin DP, Rambaut A, Gaseitsiwe S, von Gottberg A, de Oliveira T.
699 2022. Rapid epidemic expansion of the SARS-CoV-2 Omicron variant in southern Africa.
700 Nature 603:679–686.

701 71. Harari S, Tahor M, Rutsinsky N, Meijer S, Miller D, Henig O, Halutz O, Levytskyi K, Ben-Ami R,
702 Adler A, Paran Y, Stern A. 2022. Drivers of adaptive evolution during chronic SARS-CoV-2
703 infections. Nat Med 28:1501–1508.

704 72. Carabelli AM, Peacock TP, Thorne LG, Harvey WT, Hughes J, COVID-19 Genomics UK
705 Consortium, Peacock SJ, Barclay WS, de Silva TI, Towers GJ, Robertson DL. 2023. SARS-CoV-
706 2 variant biology: immune escape, transmission and fitness. Nat Rev Microbiol 1–16.

707 73. Lai AC, Chambers TM, Holland RE, Morley PS, Haines DM, Townsend HG, Barrandeguy M.
708 2001. Diverged evolution of recent equine-2 influenza (H3N8) viruses in the Western
709 Hemisphere. Arch Virol 146:1063–1074.

710 74. He W, Li G, Wang R, Shi W, Li K, Wang S, Lai A, Su S. 2019. Host-range shift of H3N8 canine
711 influenza virus: a phylodynamic analysis of its origin and adaptation from equine to canine
712 host. Vet Res 50:87.

713 75. Gahan J, Garvey M, Asmah Abd Samad R, Cullinane A. 2019. Whole Genome Sequencing of
714 the First H3N8 Equine Influenza Virus Identified in Malaysia. Pathogens 8:E62.

715 76. Olguin-Perglione C, Barrandeguy ME. 2021. An Overview of Equine Influenza in South
716 America. Viruses 13:888.

717 77. Lee K, Pusterla N, Barnum SM, Lee D-H, Martínez-López B. 2022. Investigation of cross-
718 regional spread and evolution of equine influenza H3N8 at US and global scales using
719 Bayesian phylogeography based on balanced subsampling. Transbound Emerg Dis
720 69:e1734–e1748.

721 78. Motoshima M, Okamatsu M, Asakura S, Kuribayashi S, Sengee S, Batchuluun D, Ito M,
722 Maeda Y, Eto M, Sakoda Y, Sodnomdarjaa R, Kida H. 2011. Antigenic and genetic analysis of

723 H3N8 influenza viruses isolated from horses in Japan and Mongolia, and imported from
724 Canada and Belgium during 2007-2010. Arch Virol 156:1379–1385.

725 79. Moloney B. 2011. Overview of the epidemiology of equine influenza in the Australian
726 outbreak. Australian Veterinary Journal 89:50–56.

727 80. Zhu H, Damdinjav B, Gonzalez G, Patrono LV, Ramirez-Mendoza H, Amat JAR, Crispell J, Parr
728 YA, Hammond T-A, Shiilegdamba E, Leung YHC, Peiris M, Marshall JF, Hughes J, Gilbert M,
729 Murcia PR. 2019. Absence of adaptive evolution is the main barrier against influenza
730 emergence in horses in Asia despite frequent virus interspecies transmission from wild
731 birds. PLoS Pathog 15:e1007531.

732 81. McCrone JT, Lauring AS. 2018. Genetic bottlenecks in intraspecies virus transmission. Curr
733 Opin Virol 28:20–25.

734 82. Sigal D, Reid JNS, Wahl LM. 2018. Effects of Transmission Bottlenecks on the Diversity of
735 Influenza A Virus. Genetics 210:1075–1088.

736 83. Feng KH, Gonzalez G, Deng L, Yu H, Tse VL, Huang L, Huang K, Wasik BR, Zhou B, Wentworth
737 DE, Holmes EC, Chen X, Varki A, Murcia PR, Parrish CR. 2015. Equine and Canine Influenza
738 H3N8 Viruses Show Minimal Biological Differences Despite Phylogenetic Divergence. J Virol
739 89:6860–6873.

740 84. Weaver S, Shank SD, Spielman SJ, Li M, Muse SV, Kosakovsky Pond SL. 2018. Datamonkey
741 2.0: A Modern Web Application for Characterizing Selective and Other Evolutionary
742 Processes. Mol Biol Evol 35:773–777.

743 85. Na W, Song M, Yeom M, Park N, Kang B, Moon H, Jeong D-G, Kim J-K, Song D. 2015.
744 Inefficient transmissibility of NS-truncated H3N8 equine influenza virus in dogs. J Microbiol
745 Biotechnol 25:317–320.

746 86. Feng KH, Sun M, Iketani S, Holmes EC, Parrish CR. 2016. Comparing the functions of equine
747 and canine influenza H3N8 virus PA-X proteins: Suppression of reporter gene expression
748 and modulation of global host gene expression. Virology 496:138–146.

749 87. Amat JAR, Patton V, Chauché C, Goldfarb D, Crispell J, Gu Q, Coburn AM, Gonzalez G, Mair
750 D, Tong L, Martinez-Sobrido L, Marshall JF, Marchesi F, Murcia PR. 2021. Long-term
751 adaptation following influenza A virus host shifts results in increased within-host viral
752 fitness due to higher replication rates, broader dissemination within the respiratory
753 epithelium and reduced tissue damage. PLoS Pathog 17:e1010174.

754 88. Lee K, Pusterla N, Barnum SM, Lee D-H, Martínez-López B. 2022. Genome-informed
755 characterisation of antigenic drift in the haemagglutinin gene of equine influenza strains
756 circulating in the United States from 2012 to 2017. Transbound Emerg Dis 69:e52–e63.

757 89. Dhanasekaran V, Sullivan S, Edwards KM, Xie R, Khvorov A, Valkenburg SA, Cowling BJ, Barr
758 IG. 2022. Human seasonal influenza under COVID-19 and the potential consequences of
759 influenza lineage elimination. Nat Commun 13:1721.

760 90. Rivailler P, Perry IA, Jang Y, Davis CT, Chen L-M, Dubovi EJ, Donis RO. 2010. Evolution of
761 canine and equine influenza (H3N8) viruses co-circulating between 2005 and 2008. Virology
762 408:71–79.

763 91. US Travel Association. (July 5, 2022). International travel spending in the United States
764 from 2019 to 2021, with a forecast until 2026 (in billion U.S. dollars) [Graph]. In Statista.
765 Retrieved February 16, 2023, from
766 https://www.statista.com/statistics/207083/international-travel-spending-in-the-us-since-
767 2002/.

768 92. Hannant D, Mumford JA, Jessett DM. 1988. Duration of circulating antibody and immunity
769 following infection with equine influenza virus. Vet Rec 122:125–128.

770 93. Wasik BR, Voorhees IEH, Barnard KN, Alford-Lawrence BK, Weichert WS, Hood G, Nogales A,
771 Martínez-Sobrido L, Holmes EC, Parrish CR. 2019. Influenza Viruses in Mice: Deep
772 Sequencing Analysis of Serial Passage and Effects of Sialic Acid Structural Variation. J Virol
773 93:e01039-19.

774 94. Mitchell PK, Cronk BD, Voorhees IEH, Rothenheber D, Anderson RR, Chan TH, Wasik BR,
775 Dubovi EJ, Parrish CR, Goodman LB. 2021. Method comparison of targeted influenza A virus
776 typing and whole-genome sequencing from respiratory specimens of companion animals. J
777 Vet Diagn Invest 33:191–201.

778 95. Bao Y, Bolotov P, Dernovoy D, Kiryutin B, Zaslavsky L, Tatusova T, Ostell J, Lipman D. 2008.
779 The influenza virus resource at the National Center for Biotechnology Information. J Virol
780 82:596–601.

781 96. Martin DP, Murrell B, Golden M, Khoosal A, Muhire B. 2015. RDP4: Detection and analysis
782 of recombination patterns in virus genomes. Virus Evol 1:vev003.

783 97. Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high
784 throughput. Nucleic Acids Res 32:1792–1797.

785 98. Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010. New
786 algorithms and methods to estimate maximum-likelihood phylogenies: assessing the
787 performance of PhyML 3.0. Syst Biol 59:307–321.

788 99. Rambaut A, Lam TT, Max Carvalho L, Pybus OG. 2016. Exploring the temporal structure of
789 heterochronous sequences using TempEst (formerly Path-O-Gen). Virus Evol 2:vew007.

790 100. Suchard MA, Lemey P, Baele G, Ayres DL, Drummond AJ, Rambaut A. 2018. Bayesian
791 phylogenetic and phylodynamic data integration using BEAST 1.10. Virus Evol 4:vey016.

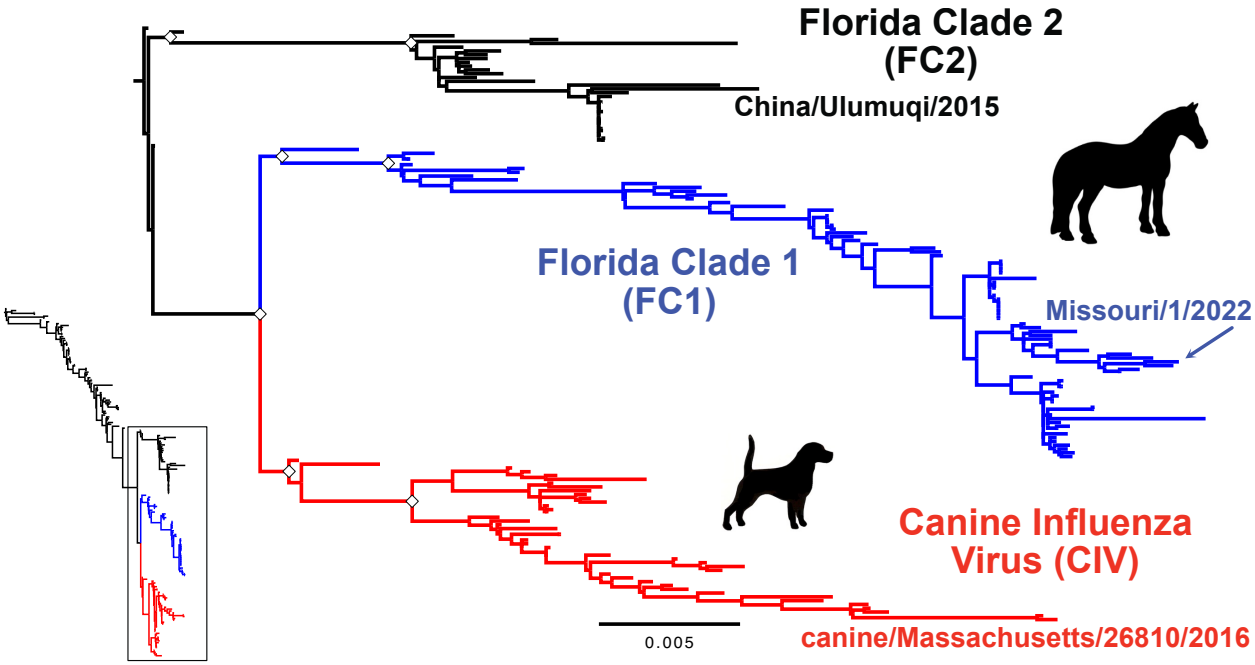
792 101. Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA. 2018. Posterior Summarization
793 in Bayesian Phylogenetics Using Tracer 1.7. Syst Biol 67:901–904.

794 102. Westgeest KB, Russell CA, Lin X, Spronken MIJ, Bestebroer TM, Bahl J, van Beek R,
795 Skepner E, Halpin RA, de Jong JC, Rimmelzwaan GF, Osterhaus ADME, Smith DJ, Wentworth
796 DE, Fouchier RAM, de Graaf M. 2014. Genomewide analysis of reassortment and evolution
797 of human influenza A(H3N2) viruses circulating between 1968 and 2011. J Virol 88:2844–
798 2857.

799

800 **Figures and Legends**

801



802

803 **Figure 1**. Recent evolution of H3N8 influenza virus. Equine influenza virus (EIV) H3N8 evolution

804 from 1963 to present has resulted in three distinct clades post 2002 (inset). ML tree of the whole

805 genome data set revealing three distinct clades: the Florida Clade 1 (FC1) and Florida Clade 2

806 (FC2) in horses, and Canine Influenza Virus (CIV) in dogs. Tree was rooted to pre-clade (preC)

807 genome Kentucky/5/2002. Scale denotes nucleotide divergence. White diamonds at major early

808 nodes represent bootstrap support >99%. Most recent genome of each clade is noted.

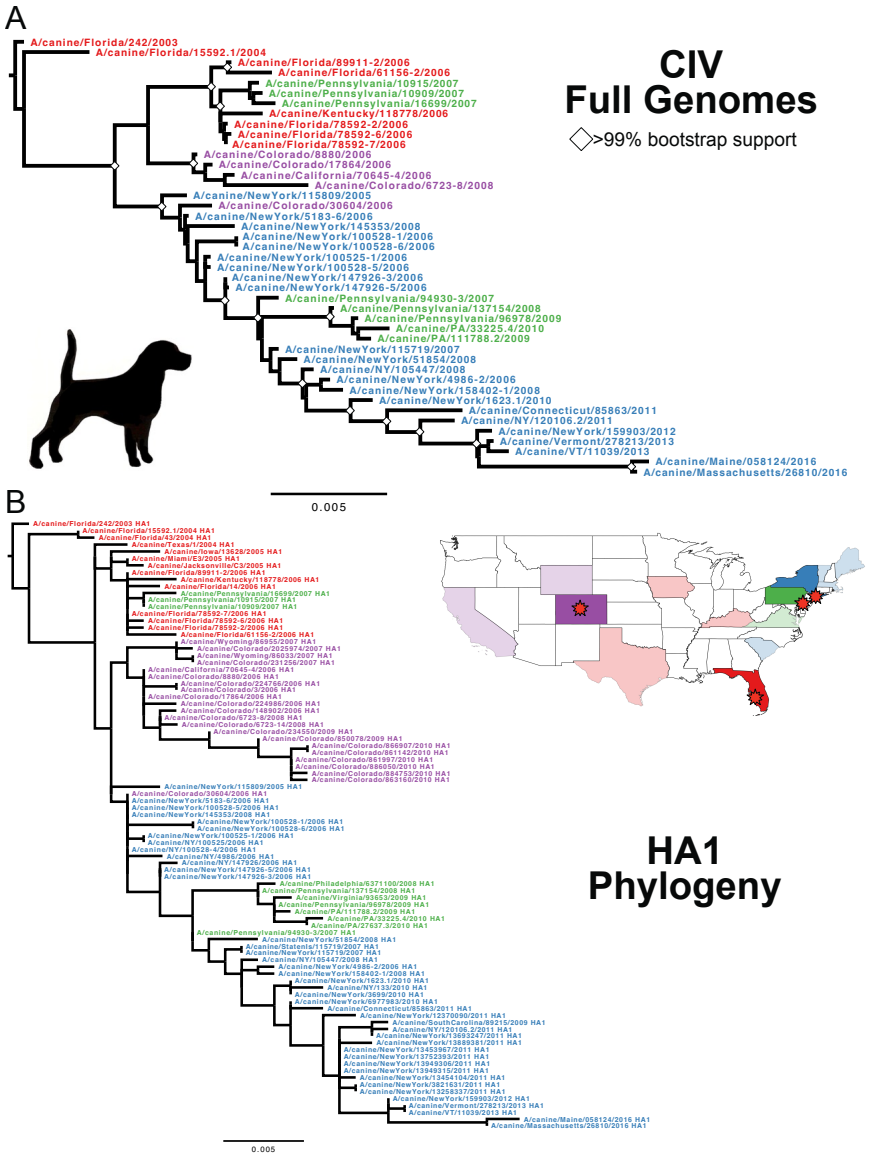


Figure 2. Evolution of the CIV H3N8 clade. (A) ML tree of the full genome data set of CIV, with taxa color defined by outbreak source region: Red, Florida/SE; Blue, New York City; Green, Philadelphia; Purple, Colorado. Tree was rooted to earliest sample, Florida/242/2003. Scale denotes nucleotide divergence. White diamonds at major nodes represent bootstrap support >99%. (B) ML tree of expanded HA1 data set reveals the strong geographic organization of subclades.

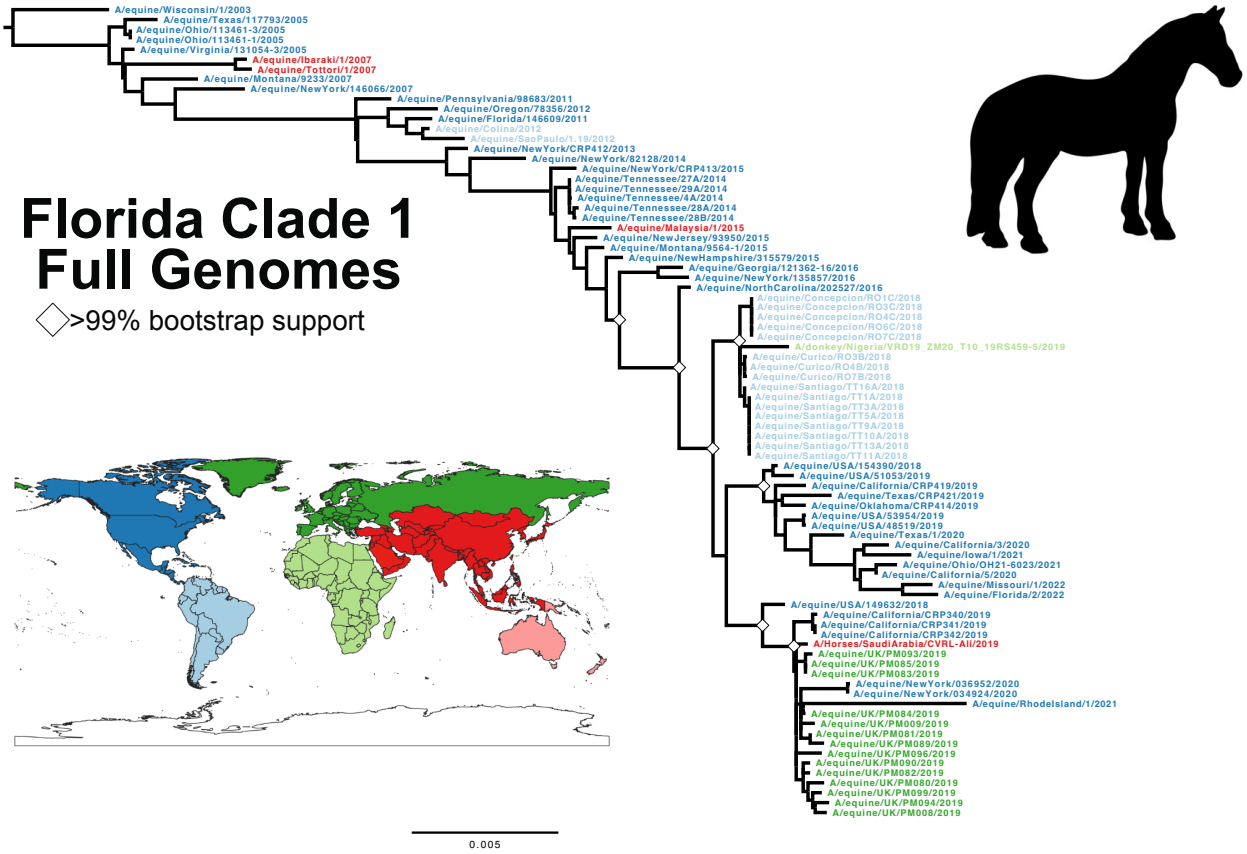


Figure 3. Evolution of the H3N8 EIV clade FC1. ML tree of full genome data set of FC1, with taxa color defined by worldwide region. Tree was rooted to earliest sample, Wisconsin/1/2003. Scale denotes nucleotide divergence. White diamonds at major nodes represent bootstrap support >99%.

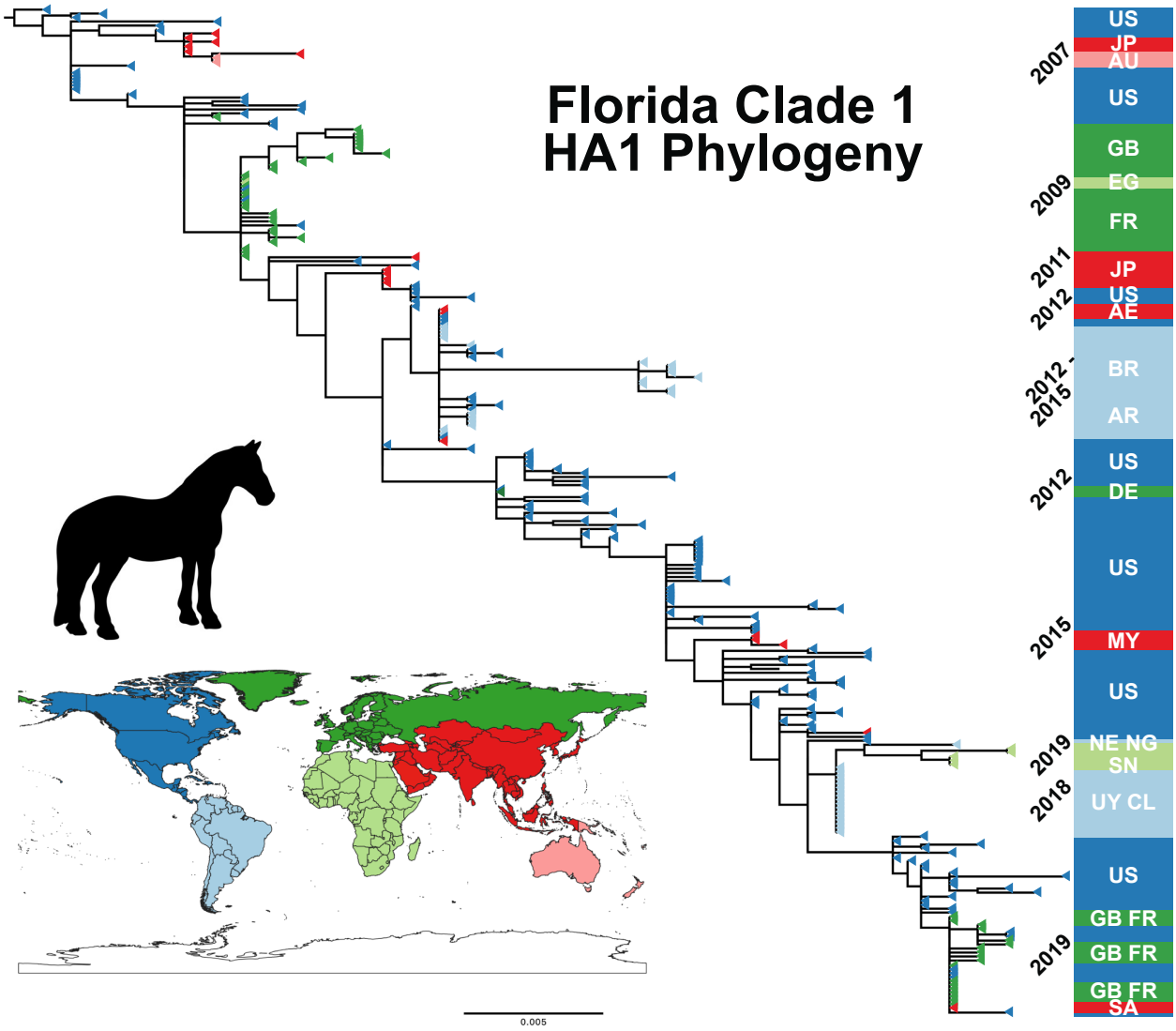


Figure 4. Evolution of the H3N8 EIV clade FC1. ML tree of the expanded HA1 data set (n=251), also colored by regional isolate. Tree was rooted to earliest sample, Wisconsin/1/2003. Scale denotes nucleotide divergence. Dates of and geography of regional subclades shows the recurring impact of US circulation on international transfers. Country ISO codes used: AE, United Arab Emirates; AR, Argentina; AU, Australia; BR, Brazil; CL, Chile; DE, Germany; EG, Egypt; FR, France; GB, United Kingdom; JP, Japan; MY, Malaysia; NE, Niger; NG, Nigeria; SA, Saudi Arabia; SN, Senegal; US, United States, UY, Uruguay.

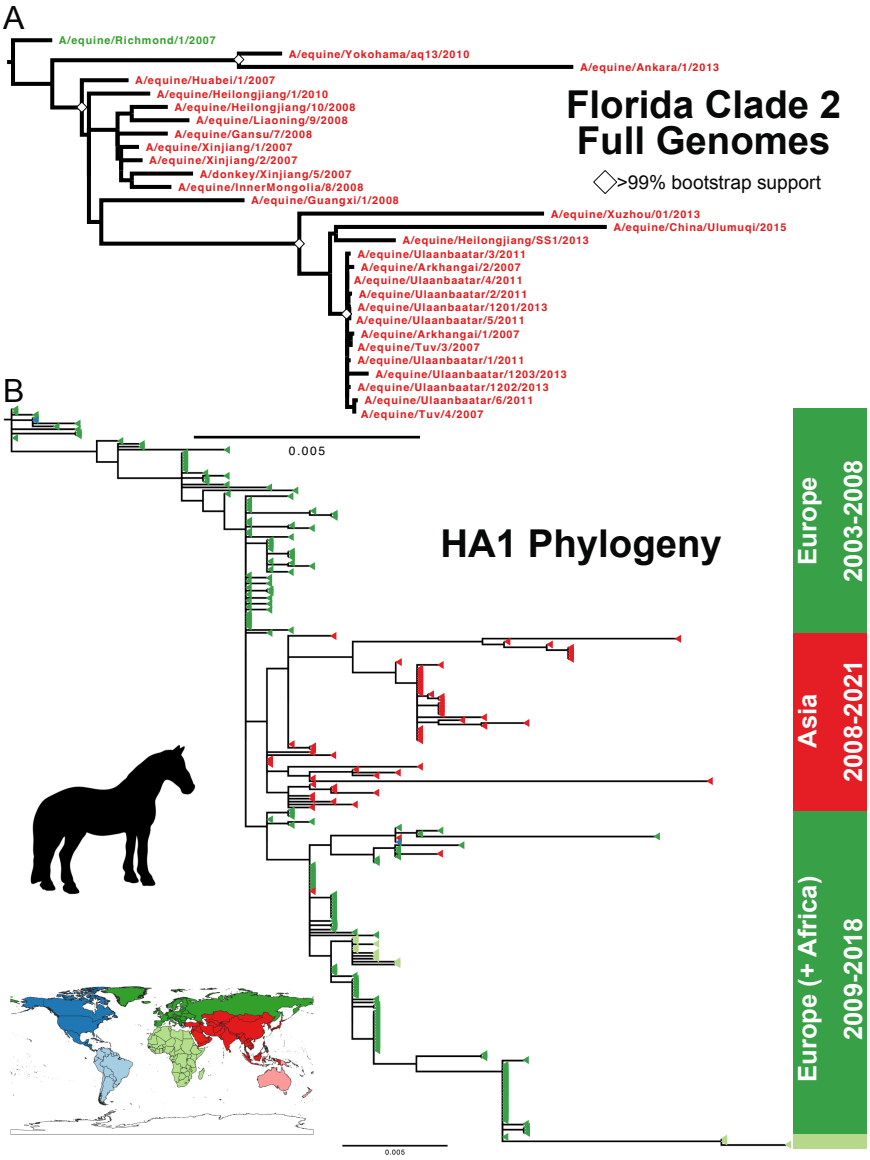
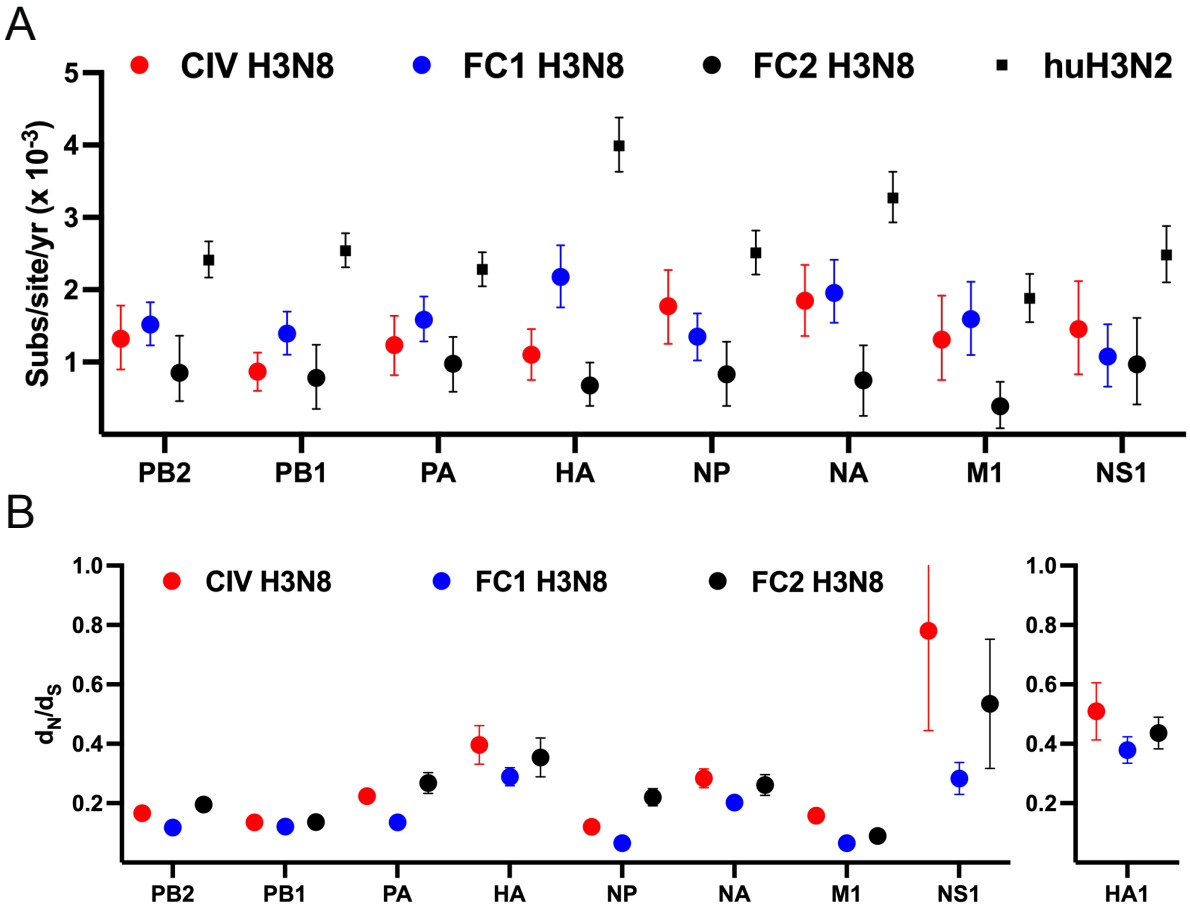


Figure 5. Evolution of the H3N8 EIV clade FC2. (A) ML tree of full genome data set of FC2, with taxa color defined by worldwide region. Tree was rooted to earliest full genome sample, Richmond/1/2007. Scale denotes nucleotide divergence. White diamonds at major nodes represent bootstrap support >99%. (B) ML tree of the expanded HA1 data set (n=254), also colored by regional origin. Tree was rooted to earliest HA1 FC2 sample, NewMarket/5/2003. Dates of and geography of regional subclades show that FC2 is now centered in mainland Asia.

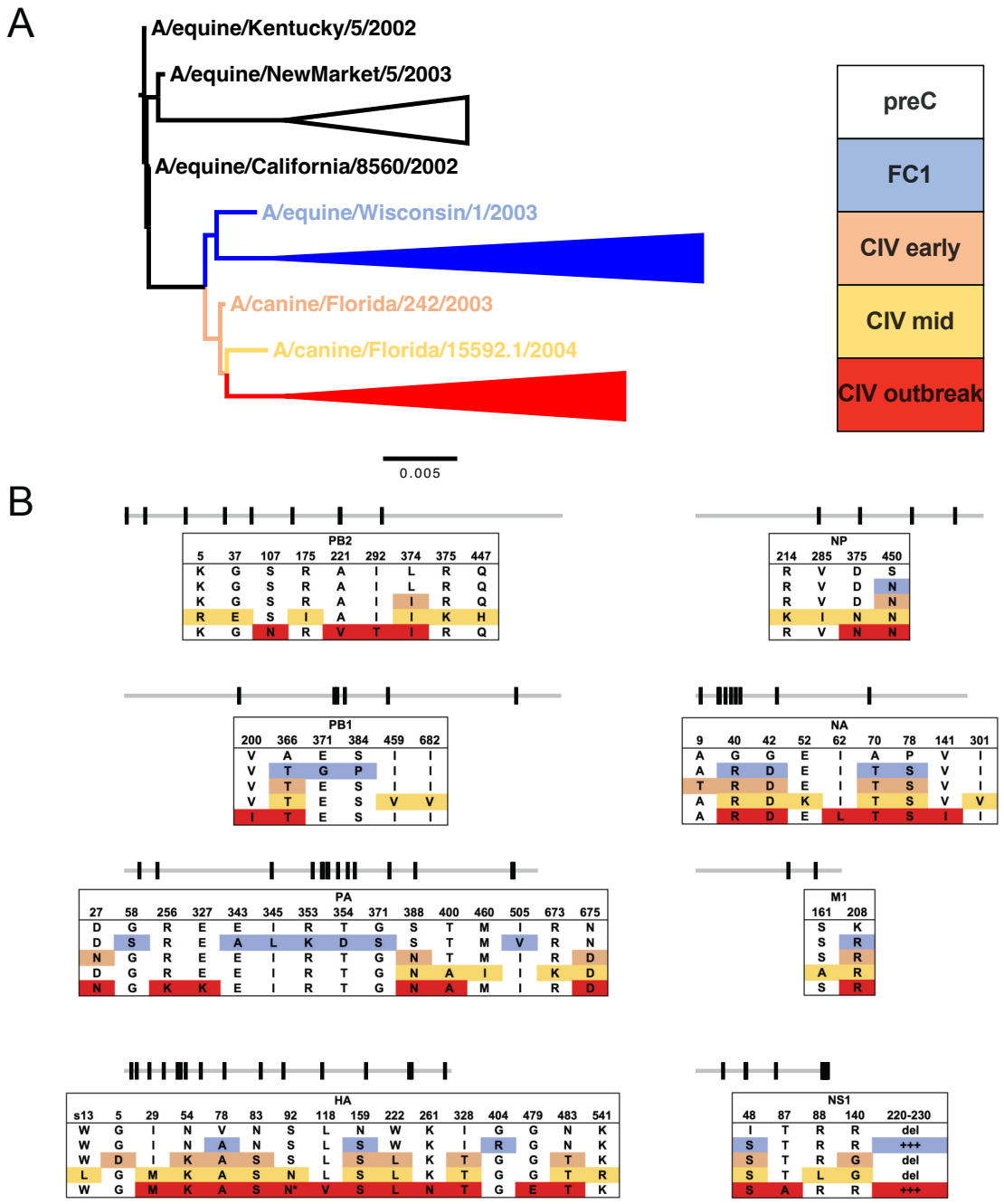
840



841

842 **Figure 6.** Molecular evolution of the H3N8 clades. (A) The nucleotide substitution rate for each
843 segment major ORF in each clade are compared to known huH3N2 reference data set (102).
844 Datapoints are mean values and error bars represent 95% highest posterior density (HPD)
845 values. (B) The mean d_N/d_S for each segment major ORF was determined using the SLAC
846 program from the Datamonkey platform. Datapoints are mean values and error bars represent
847 95% confidence intervals (CI).

848



849

850 **Figure 7**. Early molecular evolution of H3N8 clade divergence and CIV emergence. (A) The
851 H3N8 whole genome ML tree focused on the earliest preC, FC1, and CIV taxa for comparative
852 alignment. (B) Key nonsynonymous changes in the earliest diverging taxa are identified by
853 genome segment and position. Some late CIV mutations (*) are not present in all outbreak
854 subclades.