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

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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 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 clarify 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 both 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, viruses, 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)

55 **Introduction**

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

64 Most serotypes of influenza A virus (IAV) are well established in their reservoir hosts,
65 mainly birds living in aquatic environments (5). However, several serotypes appear to have a
66 relatively high propensity for infecting other animal hosts, including domestic poultry, humans,
67 swine, ferrets, seals, horses, and most recently dogs (5, 6). Most spillovers cause short-term
68 outbreaks that rapidly die out, while some have transitioned from emergent to epidemic or
69 pandemic pathogens in their mammalian hosts. In human populations, some IAV subtypes
70 (primarily H1Nx and H3Nx in the modern era) have become endemic diseases. IAV are
71 members of the family *Orthyomyxoviridae*, and have a negative-sense RNA genome arranged
72 as eight segments: PB2, PB1, PA, HA, NP, NA, M, and NS (7).

73 Horses have historically been an important mammalian host for IAV, likely including
74 epidemics in the 1870s in North America that posed a significant burden on animal health, as
75 well as on a human society that was highly dependent on horses (8). In the 20th century, horses
76 were infected by an H7N7 virus that was first recognized in 1956 and became extinct in the late
77 1970s (9). H3N8 equine influenza virus (EIV) was first isolated in 1963 in Florida, likely after
78 being introduced along with infected horses from South America, and descendants of that virus
79 continue to circulate in horses today (10–13). The emergence of circulating reassortants of
80 H7N7 and H3N8 EIV in the 1970s suggest that H3N8 contributed to subtype displacement and

extinction. Vaccines against H3N8 EIV were introduced in the mid-1970s, and (probably along with other factors) appeared to reduce the diversity and spread of the virus in the late 1970s and early 1980s, and may also have selected additional antigenic variation (11, 14). Around the year 2000 the H3N8 EIV population divided into different lineages: the Florida Clade 1 (FC1) was primarily in North America but also observed in outbreak in other regions of the world, while the Florida Clade 2 (FC2) was found primarily in Europe. Despite their names being associated with Florida, USA, both virus clades have spread to many different countries and continents (15–20). However, these subclades appear to have kept some degree of geographic separation, and they do not appear to have been in direct competition (21).

EIV is a common respiratory tract infection in horses and closely related equids, causing influenza-like signs similar to those seen in humans (22). Vaccines for EIV have been available since the 1970s which include antigens from the most commonly circulating strains, and those are widely used in domesticated horses (23). While human influenza vaccines are updated frequently with the most widespread antigenic variants, EIV vaccines are updated less often. It is unclear to what degree low vaccine effectiveness or antigenic mismatch have contributed to recently observed ‘breakthrough’ outbreaks (20, 24–27). Recommendations for vaccine formulations are regularly proposed by the World Organization for Animal Health (formerly Office International des Epizooties, OIE) expert surveillance panel (28), but strain inclusion varies in different vaccine formulations (29). The potential for horses to be sources of zoonotic disease given their close association with humans makes EIV an identified neglected disease threat of concern (30, 31). Experimental inoculation of human volunteers in the 1960s showed that the virus was able to replicate in humans with sufficient exposure (32, 33). Only through proper surveillance and sequencing of circulating EIV in different populations will a clearer picture of the virus antigenic variation be obtained, enabling the production of vaccines with a better antigenic match and greater protective efficacy. Determining the relationships between viruses causing different outbreaks through surveillance will also reveal epidemiological

107 pathways that arise during epidemics, allowing better strategies for control and perhaps
108 eradication.

109 A single H3N8 EIV transferred to dogs around 1999 to create the first known epidemic of
110 canine influenza virus (CIV). Molecular and epidemiological evidence suggests the epidemic
111 originated from a single, cross-species transmission event of a non-reassortant H3N8 EIV, with
112 cases being initially identified in greyhounds in a training facility in Florida in 2003 and 2004
113 (34–37). The H3N8 CIV spread to several regions of the USA around this period, likely due to
114 transport of infected greyhounds, although the virus only achieved prolonged transmission in
115 dogs within larger dense populations, including animal shelters, kennels, or canine day cares
116 (38). The virus appears to have persisted in large metropolitan areas (e.g. Denver and Colorado
117 Springs, New York City, and Philadelphia) with large shelter dog populations that had high
118 standing populations and high rates of turnover (39). While the CIV H3N8 emerged to cause an
119 epidemic, other observed spillovers of EIV H3N8 into dogs (in 2002 in the UK and 2007 in
120 Australia) did not result in onward canine transmission (40, 41). Domesticated canine
121 interactions are one of the most dramatic cross-species associations with humans, placing their
122 infectious diseases as critical potential sources of risk. No observed human infections with
123 H3N8 CIV were observed (42). A second CIV arose around 2005 in Asia, and that H3N2 strain
124 appears to have been derived directly from an avian influenza virus that transferred to dogs
125 (43). H3N2 CIV appears to have circulated widely in Korea and China for a number of years,
126 and has been repeatedly introduced into the USA and Canada where it has mostly caused local
127 and self-limiting outbreaks (44). Risk assessment of H3N2 CIV to humans was found to be
128 minimal (45).

129 Host transfers underpin many infectious disease epidemics or pandemics, and in recent
130 years such events have given rise to new emerging viruses in humans and other animals,
131 including the 2009 pandemic swine influenza (pdmH1N1), Ebolavirus, SARS-CoV-1 and -2, and
132 the H3N8 and H3N2 CIV (1, 46). Influenza surveillance networks (such as the World Health

133 Organization Global Influenza Surveillance & Response System, WHO GISRS) have made
134 considerable leaps in the past decade, with rapid identification of human cases of highly
135 pathogenic avian influenza (47, 48). Despite this, gaps remain in our ability to predict changes in
136 host-range that result in virus emergence, or to create early warning systems to detect and
137 mitigate outbreaks before they get underway (4, 49, 50). Strategies for controlling emerging
138 viruses primarily involve aggressively managing outbreaks after they first occur. Yet, this
139 requires a clear understanding of the routes of transmission, ongoing circulation, and likely host
140 adaptation leading to increased spread or immune evasion, and such knowledge is often lacking
141 in the early stages of outbreaks (3). It is also often unclear whether and how virus evolution in
142 the original host facilitates the initial successful spillover infection, or how the viruses later
143 evolve and adapt to their new hosts compared to evolution that occurs in their original hosts.
144 Understanding these processes in more detail may allow us to anticipate and forestall the
145 emergence of other viruses in the future.

146 As well as local outbreaks, a defining feature of the most threatening virus emergence
147 events is longer distance and international transfers that allow viral outbreaks to sustain over
148 time and develop into pandemics, particularly within human host populations (51, 52). Long-
149 distance transfers are also a feature of H3N8 EIV epidemiology, likely associated with the
150 movement of infected racing and sport horses (26, 53, 54). H3N2 CIV similarly has transferred
151 between continents (44). In contrast, the H3N8 CIV outbreaks were more confined, circulating
152 within and between animal shelters and kennels within the central and eastern regions in the
153 United States (38, 39). Notably, that virus lineage did not appear to spread outside the USA.

154 Modern genomic epidemiology has allowed the detailed examination of viral outbreaks,
155 including the spread and replacement of viral variants (2, 55–57). For example, the emergence
156 of more transmissible or antigenic variant viruses that displace earlier lineages is commonly
157 seen in evolving virus populations, including 2009 pdmH1N1, SARS-CoV-2 (B.1.1.7 'Alpha', or
158 B.1.1.529 'Omicron'), and the canine parvovirus type-2a variant that emerged and efficiently

159 replaced the earlier strains of that virus (58–64). These events highlight the importance of
160 continued surveillance and analysis of viruses; both in new hosts and in their original hosts, as
161 such comparative analysis allows us to identify mechanisms of true adaptive selection following
162 emergence.

163 To resolve the evolution of H3N8 in dogs and horses we collected genomic data from
164 our own sequencing and public repositories of H3N8 beginning around 2001. Complete genome
165 sequences of all segments enable phylogenetic relationships and host-specific evolution to be
166 determined. Here we investigate the emergence the H3N8 canine influenza virus (CIV) and its
167 origin from the H3N8 equine influenza virus (EIV), describe the later extinction for CIV while also
168 following the continuing evolution and epidemiology of EIV among horses in several geographic
169 regions of the world.

170

171 **Results**

172 **H3N8 experienced major clade divergence in the early 2000s, including the**
173 **emergence of CIV in dogs.** Despite years of spread and evolution in horses since its first
174 emergence around 1963, H3N8 EIV diversity had largely collapsed to a single North American
175 clade by the end of the 20th century (Figure 1, inset) (15, 65). This 'Florida' clade then gave rise
176 to the FC1 and FC2 subclades. A full genome phylogeny since 2001 shows the presence of pre-
177 clade divergence viruses (preC), and viruses within the monophyletic FC2, FC1, and CIV
178 groups. Divergence occurred roughly after the appearance of Kentucky/5/2002,
179 California/8560/2002, and Newmarket/5/2003 preC (Figure 1), so that the first full-genome
180 samples from the FC2, FC1 and CIV clades are Richmond/1/2007, Wisconsin/1/2003,
181 ca/Florida/242/2003, respectively. These FC2 and FC1 sequences are currently (or closely
182 match) the EIV formulations frequently manufactured and used for vaccination (29).

183 We further gathered all detailed collection dates at best accuracy (YYYY-MM-DD) to
184 review the temporal nature of the data set. A root-to-tip regression analysis of the full genome

185 data set revealed strong clock-like structure (R^2 = 0.92) and an evolutionary rate of 1.5 x 10^{-3}
186 nucleotide substitutions/site/year (Figure S1A). However, due to an expanded FC2 data set pre-
187 2007 the HA1 data set showed a bi-modal clustering (Figure S1B). The establishment and
188 persistence of these lineages allows a direct comparison of H3N8 influenza virus evolution in
189 novel (dog) and original (horse) hosts following the host transfer and emergence event giving
190 rise to CIV. To determine what genomic characteristics may have allowed FC1 (and FC2) to
191 persist in nature, while CIV went extinct, we analyzed the CIV, FC1 and FC2 clades since their
192 divergence in parallel (Figure 1).

193 **CIV only circulated in the United States and went extinct around 2016**. The last
194 known positive cases of H3N8 CIV were reported in the Northeast USA in 2016, two samples of
195 which (ME and MA) were sequenced shortly thereafter by our group. No further H3N8 positive
196 cases in the USA have been reported, even though this was a time of frequent canine influenza
197 testing due to the emergence and spread of H3N2 CIV in the US in 2015 and 2016 (44).

198 We gathered a data set of 43 complete CIV genomes (and an expanded 88 HA1 reading
199 frame sequences) and employed the same methods as for the full H3N8 data set. Our full
200 genome CIV phylogeny (Figure 2A) closely matches the monophyletic group seen in the larger
201 H3N8 full genome data set (Figure 1) and previous analyses (66). The full genomes of CIV
202 showed a strong clock-like evolution (R^2 = 0.94) at approximately the same rate (1.8 x 10^{-3}
203 substitutions/site/year, Figure S1C) as observed with the larger H3N8 full genome data set. We
204 used the expanded HA1 data set to connect the phylogenetic tree to the geographic data
205 (Figure 2B). Subclades of CIV were highly structured by regional introduction and spread,
206 mostly localizing to outbreak centers in Florida, New York City, Philadelphia, and the cities of
207 Colorado. Secondary geographic sites appear to have involved secondary outbreaks that were
208 related by phylogenetics back to their source locations.

209 We further inferred the demographic history of these populations using Bayesian
210 analysis with a Skyline Plot (BSP) tree prior, enabling us to estimate changes in genetic

211 diversity – a measure of effective population size (N_e) – through time (Figures S3-5). For both
212 data sets we found that N_e plots correspond to virus natural history and epidemiology: large
213 population and diversity growth occurring during the initial US-wide outbreaks of 2005-2007,
214 followed by regional die out and the eventual complete extinction (Figures S2A-B).

215 **EIV FC1 is a US-centric virus that contributes to international transfers and**
216 **outbreaks**. We compiled 82 FC1 full genome and 252 HA1 sequence data sets including those
217 newly generated here. The FC1 full genome phylogeny shows a fairly linear clade divergence
218 (Figure 3). The trees show strong signatures of geographic clustering, suggesting frequent
219 single-introduction outbreaks in particular locations including South America, Europe, and other
220 regions. There is a strong clock-like evolution (R^2 = 0.99) at approximately the same rate (1.7 x
221 10^{-3} substitutions/site/year, Figure S1E) seen in the larger H3N8 full genome data set and the
222 CIV clade. We again estimated N_e changes through time, and while sampling bias and poor
223 admixture limits making broad conclusions, we were able to identify key demographic transitions
224 and data gaps (Figures S2A, S6, S7).

225 The phylogenetic tree of our expanded HA1 sequences largely paralleled the full-length
226 genome tree, further clarifying the larger geographic movements of FC1 since its divergence.
227 The overall clade structure reveals an apparent US-based continuous circulation that seeds
228 multiple international transfers and outbreaks in different regions of the world (Figure 4). Some
229 of these historical transfers have been reported and analyzed previously and confirmed here
230 (18, 67–71). Our Bayesian demographic analysis of the FC1 HA1 data set (Figure S8) also
231 shows that the effective population size largely mirrors the full-length genome data set, yet with
232 more points of transition and changes in diversity, some of which are likely revealed by the
233 increased sampling resolution (Figure S2B).

234 Recent FC1 phylogenetic structure (in both full genome and HA1 data sets) reveals two
235 closely related subclades at this point in time: the viruses circulating in the US, and those
236 collected from the 2019 European outbreak. The directions of transmission appear complex, but

237 since the virus in the United States was circulating for a few years prior to 2019, that was likely
238 transferred from the US to the United Kingdom and elsewhere in Europe. A few viruses
239 collected in California in 2019 clustered with the European subclade, likely in horses that had
240 been imported from Europe (personal communication). Some 2020-2021 US cases in the
241 Northeastern states of New York and Rhode Island also more closely match the UK samples,
242 suggesting either parallel evolution of US viruses, or additional transfers from Europe back to
243 the USA. Further sampling in the USA, particularly from the Northeast region, is needed to
244 resolve this question.

245 **FC2 represents an under sampled and likely limited clade of EIV now only**
246 **circulating in some regions of Asia**. The public repositories have limited numbers of full
247 genomes from the FC2 clade, but there are more HA1 sequences. Sampling appears to be
248 relatively sparce, with multiple samples and sequences from notable outbreaks, so that there is
249 extensive genetic redundancy among the samples available. Our FC2 full genome ML tree
250 showed no variation from the full H3N8 data set analysis (Figure 5A, Figure 1). The more
251 substantial HA1 data set (n=254, Figure 5B) clearly revealed geographic distribution of recent
252 FC2 samples: while FC2 were centered in Europe between around 2000 and 2014, the clade
253 appears to have been transferred to Asia around 2009. Europe has seen fewer FC2 cases, with
254 last reports in 2017, while since 2018 only a single subclade has been present in mainland Asia.
255 The small number of full FC2 genomes (Figure S1G-H) resulted in Bayesian analyses
256 characterized by high statistical uncertainty (Figures S2A-B, S9-11).

257 We also note that FC2 isolates have been identified in various equid species (Mongolian
258 horses, Arabian horses, Donkeys), as well as dogs, although their epidemiological relevance is
259 unknown. Horses in Asia appear to be regularly exposed to avian influenza but have generally
260 failed to support a new emergent strain due to a lack of adaptation (72).

261 **H3N8 clades show distinct mutational signatures reflecting host biology and**
262 **unique epidemiology**. We next compared H3N8 evolution in different hosts to see if there were

263 obvious signatures of host-specific evolution after transfer to, and sustained transmission in,
264 dogs. The rate of genome evolution (i.e. nucleotide substitutions per site per year for each
265 segment) for each clade showed that substitution rates in CIV and FC1 were essentially the
266 same in all segments except HA (Figure 6A), suggesting that if new-host adaptation was
267 present it was masked in the general background rate of genetic drift (73, 74). In contrast, the
268 HA, NA, and M1 substitution rates are significantly greater within the FC1 clade than the FC2.
269 All H3N8 clades within horses and dogs exhibit lower substitution rates than reported for
270 influenza circulating in human host populations (huH3N2, Figure 6A), particularly in HA and NA
271 where antigenic selection would be most apparent.

272 We have previously reported comparative analyses of CIV and FC1 through 2015 (75).
273 Only two CIV isolates have been generated since this time, while FC1 has continued to circulate
274 widely in different regions of the world. Here, to better identify any residues subject to selection,
275 we have updated that analysis based on the many additional the full genome sequences of the
276 different clades. To do this we utilized several approaches in the Datamonkey analytical toolset
277 (https://datamonkey.org/, (76)). Using SLAC (Single-Likelihood Ancestor Counting) we
278 measured the mean d_N/d_S ratio of each segment for each clade (Figure 6B). An elevated ratio is
279 seen in the HA ORF (particularly in the HA1 antigenic domain), but at no significant difference
280 between clades. The extensive divergent selection on the CIV and EIV H3N8 NS segment,
281 reflected in variant segment length, has previously been analyzed in detail (77). In sum, it
282 appears that FC1 is undergoing greater evolutionary change in horses than the FC2 clade, or
283 than the H3N8 CIV in dogs up until 2016.

284 **CIV early mutations suggest host adaptation to dogs, yet with no change in**
285 **epidemiology and avoidance of extinction.** The sequences of viruses spilling over into new
286 hosts will reflect the composition of the single virus that makes the initial transfer, and then will
287 likely undergo adaptation to the properties and physiology of the new host animal, including
288 optimizing onward transmission (3). We therefore compared the sequences and evolution of the

289 viruses that were closely related to the common ancestor of CIV (preC), the evolution of H3N8
290 CIV in its new host, and parallel continued evolution of the early FC1 H3N8 EIV in horses
291 (Figure 7A). This revealed multiple transitional mutations in CIV (Figure 7B), some of which
292 have been previously analyzed (35, 75, 77, 78). However, these genetic changes appear not to
293 have allowed CIV to increase its spread among the general dog populations, such that the virus
294 continued to be maintained as shelter-driven outbreaks which resulted in viral extinction, likely
295 through stochastic fade out (39). It is also likely that selection on the virus for spread within
296 dense and high turn-over shelter or kennel populations did not result in the selection of
297 mutations enabling enhanced spread in the household dog population required to develop a true
298 epidemic or pandemic (38). Despite this successful transfer of the H3N8 EIV to dogs, all other
299 spillovers have resulted in only single canine infections or short-lived local outbreaks (40).
300 It is also noteworthy that the H3N8 EIV FC1 acquired additional mutations during its
301 parallel spread in horses. There is some evidence that H3N8 may have undergone further host
302 adaptation in horses over time (79). This change in contingency of the EIV may suggest that the
303 preC consensus genome was particularly suited to clade divergence and host-switching ~1999.
304 While some antigenic selection may occur (20, 80, 81), most mutations are not in the antigenic
305 sites of the HA or NA, and likely represent genetic drift during the outbreak-driven circulation of
306 FC1 (Figure 4). Equids may be similar to humans as long-lived vertebrates where relatively high
307 levels of anti-EIV antibody immunity are induced due to either infection or vaccination but wane
308 in time between lifetime exposures (82). Discerning true antigenic evolution from background
309 genetic drift will require greater surveillance and testing of immunity in horses.
310

311 **Discussion**

312 A detailed understanding of the replication and spread of infectious diseases allows
313 effective control strategies to be devised. Influenza A viruses are important pathogens in many
314 different hosts and have repeatedly emerged and spread as pandemic respiratory pathogens in

315 humans (7). Some mammals occasionally suffer outbreaks from avian origin influenza, including
316 seals and mink notably during recent spread of highly pathogenic avian influenza (HPAI) H5N1
317 (83, 84). Other hosts, including swine, horses, and dogs have suffered epidemics from both
318 avian- and mammalian-origin influenza subtypes that led to adaptation and endemic circulation.
319 Aside from H17 and H18 in bats, IAVs all appear to derive from aquatic bird host reservoirs,
320 where they are primarily spread through fecal-oral routes (5, 6). While endemic human influenza
321 outbreaks appear to involve common global movement and widespread infections, IAVs in other
322 hosts involves more complex epidemiology, often with constrained geographic spread, and
323 many outbreaks dying out without obvious human interventions. The recent apparent extinction
324 of the Influenza B Yamagata lineage in humans suggest that influenza viruses may have a more
325 tenuous foothold in mammalian host population than has sometimes been assumed (85).
326 **Equine and canine H3N8 have different evolutionary fates.** The analysis presented
327 here focuses on the avian-mammalian emergent H3N8 viruses: first in their original equine
328 hosts (EIV) in which they have been circulating for 60 years, and then with comparisons to the
329 same virus in dogs (CIV), to understand the divergent evolution of the same virus in different
330 hosts. While EIV continues to circulate and infect horses around the world, we report here that
331 CIV was last seen in 2016 in the Northeast United States, and is apparently now extinct.
332 H3N8 CIV was likely entirely contained to the United States during its ~17 years of
333 transmission in dogs, as no cases of this lineage have been reported in other countries. Our
334 new complete genome sequencing of the H3N8 CIV isolates from throughout the course
335 epidemic show that its geographic structure arose from an initial burst of widespread disease
336 that may have been associated with the movement of infected greyhounds. After 2007, only
337 local transmission was seen in Colorado and close by in Wyoming, up to around 2010 (86). In
338 the Northeast of the USA the virus was first reported in New York in 2005 and in Pennsylvania
339 in 2007. The virus continued to circulate in New York, where it was isolated from short-lived
340 outbreaks that occurred after introduction of virus (likely due to transport of infected dogs from

341 the shelters where the virus was endemic), and those were seen in Connecticut, Pennsylvania,
342 Vermont, Maine, and Massachusetts up until 2016. Our analysis confirms that the outbreaks in
343 Colorado and the Northeast were distinct, and also that after 2012 only a single New York-
344 centered lineage of virus continuing to circulate in dogs.

345 **Equine influenza virus circulates widely in the USA and in different regions of the**
346 **world.** In contrast, the H3N8 EIV has been found throughout North America for the 60 years
347 since it was first isolated. However, frequent international transfers of the virus are also a
348 feature of its epidemiology. We confirm here that outbreaks in several countries around the
349 world were likely directly, or indirectly, caused by viruses from North America. Those included
350 widespread outbreaks in South America that started in 2018, and in the United Kingdom and
351 elsewhere in Europe starting in 2019. Similarly, viruses from Japan, Australia, Malaysia have
352 been closely related to viruses from the USA. Recent outbreaks in Western Africa also fit this
353 pattern, as viruses from Senegal, Nigeria, and Niger were closely related to those from South
354 America, which were due to viruses originating in the USA. SARS-CoV-2 drastically changed
355 human behavior during 2020 and 2021, with international travel spending being reduced by 76%
356 in 2020 compared to 2019 following international travel bans imposed by many countries (87).
357 Equine sport activities in the United States and around the world were impacted by varied
358 economic curtailment, including the international transfer of horses. Yet we see continued FC1
359 infections in the United States between 2020 and 2022, and a potential inter-relatedness
360 between US and European samples in that time, suggesting a potential return to the norm.
361 Overall, it appears that the FC1 lineage of EIV is maintained in the horse population of the
362 United States, and that provides viruses causing outbreaks in other regions of the world (69).

363 **What host differences explain the different viral evolutionary fates?** As dogs
364 usually live in households as single animals or in small groups as their population structure and
365 movement patterns likely differ from that of horses. Larger numbers of susceptible dogs may be
366 found in animal shelters, kennels, or dog day cares, and these will allow increased transmission.

367 CIVs will spread rapidly within most shelters, and then the virus will die out once the population
368 has all been infected and become immune, or transmission may be stopped within the shelter
369 by quarantining affected dogs (38, 39). Horses may be more commonly found in groups or
370 stables, giving a chance for increased transmission. Additionally, there appear to have been no
371 international transfers of dogs infected with H3N8 CIV.

372 Interestingly, the reservoir of H3N2 CIV in China and Korea appears to have been dogs
373 being bred in large numbers, with infected dogs then transported to the USA or Canada to
374 cause outbreaks among different dog populations. However, once again there has been no
375 onward transmission of that H3N2 CIV to other countries from North America, suggesting that
376 the recent US dog population structure may represent a 'dead-end' for endemic IAV circulation
377 (44).

378 **Conclusion.** Our analysis of H3N8 evolution in the horse and canine clades show
379 similar levels of variation that are lower than that observed for influenza in humans. The data
380 therefore suggest that host population structure and epidemiology rather than specific adaptive
381 evolution are driving the sustained transmission of FC1 in horses. Given the extinction of CIV
382 H3N8, this suggests that well targeted interventions could in turn result in extinction of EIV
383 H3N8 FC1 in horses. This likely requires a more refined epidemiological understanding of
384 circulation in the United States, updated vaccination formulation for FC1 and greater uptake. It
385 may further require screening of international transfers of horses for influenza to reduce
386 worldwide transmission, particularly the export of US-borne FC1 lineages, as well as the 'return'
387 of viruses to the USA from Europe. Rational human intervention may be necessary to 'break'
388 the host-structure epidemiology that is sustaining this virus.

389

390 **Materials and Methods**

391 **H3N8 Sample Collection.** Samples were obtained from various diagnostic centers
392 following routine passive diagnostic testing for respiratory disease (listed in Table S1, red).

393 Samples were received as either nasal or pharyngeal swab material, virus isolated in
394 culture/egg passages, or extracted total nucleic acid (tNA) first utilized for quantitative reverse
395 transcription-PCR (qRT-PCR) positivity. vRNA was extracted from clinical swab samples and
396 isolated using the QIAamp Viral RNA Mini kit. Purified vRNA or tNA was then either directly
397 used for influenza multi-segment RT-PCR or stored at -80˚C.

398 **Generation of Influenza Virus Sequences.** Genomes from samples were generated as
399 cDNAs using a whole genome multi-segment RT-PCR protocol as described previously (88,
400 89). A common set of primers (5′ to 3′, uni12a, GTTACGCGCCAGCAAAAGCAGG; uni12b,
401 GTTACGCGCCAGCGAAAGCAGG; uni13, GTTACGCGCCAGTAGAAACAAGG) that recognize
402 the terminal sequences of the influenza A segments were used in a single reaction with
403 SuperScript III OneStep RT-PCR with Platinum *Taq* DNA polymerase (Invitrogen). Following
404 confirmation by gel electrophoresis, viral cDNA was purified either by standard PCR reaction
405 desalting columns or with a 0.45X volume of AMPure XP beads (Beckman Coulter). Nextera XT
406 libraries were generated with 1 ng of cDNA material, while Nextera FLEX libraries were
407 generated with 150-200 ng of cDNA (Invitrogen). Libraries were multiplexed, pooled, sequenced
408 using Ilumina MiSeq 2 X 250 sequencing, and consensus sequences were determined for each
409 genome segment after assuring full genome coverage.

410 Consensus sequence editing was performed using Geneious Prime 2022.1.1. Paired
411 reads were trimmed using BBduk script (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-
412 guide/bbduk-guide/) and merged. Each sequence was assembled by mapping to a reference
413 sequence of a previously annotated equine or canine H3N8 sequence.

414 **Phylogenetic Analysis.** H3N8 nucleotide sequences were downloaded from the NCBI
415 Influenza Virus Database and combined with the consensus genomes generated in this study
416 (90). We focused on genomes from 2001 to present, encompassing the formation of the major
417 virus clades (FC2, FC1, CIV). Both inter- and intra-subtype reassortants were identified using
418 RDP4 (seven methods: RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan, 3seq) and

419 excluded if found to be a statistically significant outlier in two or more methods (91). Our total
420 data set comprised 157 full genomes (preC: 3, FC2: 29, FC1: 82, CIV: 43), with an expanded
421 611 HA1 reading frames (preC: 17, FC2: 254, FC1: 252, CIV: 88) (Tables S1-2). Sequences
422 were manually trimmed to their major open reading frames (PB2: 2280nt, PB1: 2274nt, PA:
423 2151nt, HA: 1695-1701nt, NP: 1497nt, NA: 1410-1413nt, M1: 759nt, and NS1: 654-693nt) and
424 either analyzed separately or concatenated with all other genome segments from the same
425 virus sample. HA1 nucleotide sequences were trimmed to cover amino acids 1-329 (H3
426 numbering). Nucleotide sequences were aligned using MUSCLE (92). Maximum likelihood (ML)
427 phylogenetic analysis was performed by PhyML (93), employing a general time-reversable
428 (GTR) substitution model, gamma-distributed (Γ4) rate variation among sites, and bootstrap
429 resampling. Both MUSCLE and PhyML were run under the Geneious Prime platform. ML trees
430 were visualized and annotated using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).
431 The temporal signal (i.e. molecular clock-like structure) of the data was assessed by a
432 regression of root-to-tip genetic distance against date of sampling using our ML tree and the
433 TempEst v.1.5.3 software (94). Accurate collection dating to the day (YYYY-MM-DD) was
434 utilized for all samples where this information was available. The sampling dates used for all
435 isolates are listed in Tables S1 and S2.
436 **Bayesian Phylogeny and Analysis.** We used a Bayesian Markov chain Monte Carlo
437 (MCMC) approach to better estimate virus divergence times and population dynamics. Analyses
438 were performed in BEAST v.1.10.4 (95), where MCMC sampling was performed with a strict
439 clock, a general time-reversable (GTR) substitution rate with gamma distribution in four
440 categories (Γ4), and assuming a Bayesian Skyline Plot (BSP) prior demographic model.
441 Analyses were generally performed for 100 million runs, with replicates combined in Log
442 Combiner v1.10.4. All output results were examined for statistical convergence in Tracer v1.7.2
443 (effective sample size [ESS] ≥200, consistent traces, removed burn-in at 10-15%) (96).

444 Maximum clade credibility (MCC) phylogenetic trees were generated by Tree Annotator v1.10.4,
445 and visualized by FigTree v1.4.4.

446 **Analysis of Selection Pressures.** The relative numbers of synonymous (d_S) and
447 nonsynonymous (d_N) nucleotide substitutions per site in each ORF of each segment were
448 analyzed for the signature of positive selection (adaptive evolution, $p<0.05$ or >0.95 posterior)
449 using a variety of method packages available in the Datamonkey package
450 (https://datamonkey.org/, (76)): BUSTED (**B**ranch-site **U**nrestricted **S**tatistical **T**est
451 for **E**pisodic **D**iversification), FEL (**F**ixed **E**ffects **L**ikelihood), FUBAR
452 (**F**ast, **U**nconstrained **B**ayesian **A**pp**R**oximation), MEME (**M**ixed **E**ffects **M**odel of **E**volution), and
453 SLAC (**S**ingle-**L**ikelihood **A**ncestor **C**ounting).

454 **Graphing and Statistical Analysis.** All figure graphs were generated in GraphPad
455 Prism v.9.5.0.

456 **Data Availability.** Virus genome consensus sequences have been submitted to
457 GenBank, and accession numbers of the sequences used (and generated) for this study are
458 provided in Tables S1 and S2.

459

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470

471 **Supplemental Materials**

472 TablesS1_2_Wasik2023.xlsx

473 Table S1: Full genome data set

474 Table S2: HA1 data set

475 SuppFig_Wasik2023.pdf

476 Figure S1: Clock Analysis (TempEst)

477 Figure S2: Bayesian Skyline Plots (BSPs)

478 Figure S3: CIV full genome MCC

479 Figure S4: CIV segment MCC

480 Figure S5: CIV HA1 MCC

481 Figure S6: FC1 full genome MCC

482 Figure S7: FC1 segment MCC

483 Figure S8: FC1 HA1 MCC

484 Figure S9: FC2 full genome MCC

485 Figure S10: FC2 segment MCC

486 Figure S11: FC2 HA1 MCC

487

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Figures and Legends

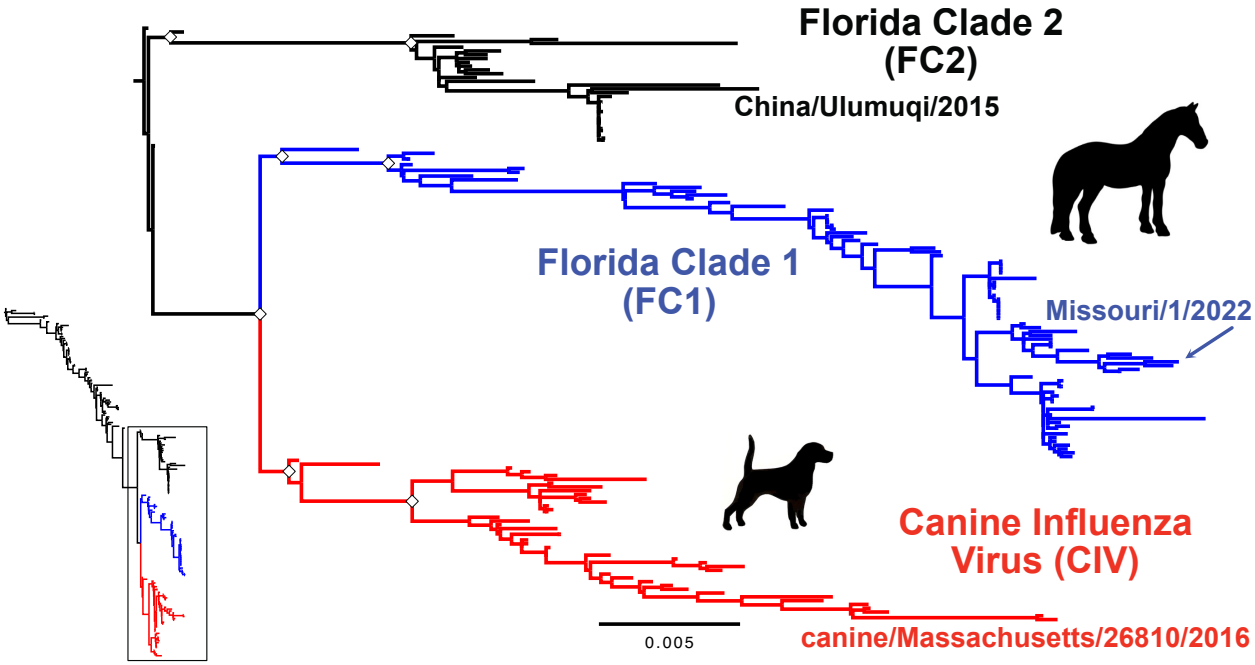

Figure 1. Recent evolution of H3N8 influenza virus. Equine influenza virus (EIV) H3N8 evolution from 1963 to present has resulted in three distinct clades post 2002 (inset). ML tree of the whole genome data set revealing three distinct clades: the Florida Clade 1 (FC1) and Florida Clade 2 (FC2) in horses, and Canine Influenza Virus (CIV) in dogs. Tree was rooted to pre-clade (preC) genome Kentucky/5/2002. Scale denotes nucleotide divergence. White diamonds at major early nodes represent bootstrap support >99%. Most recent genome of each clade is noted.

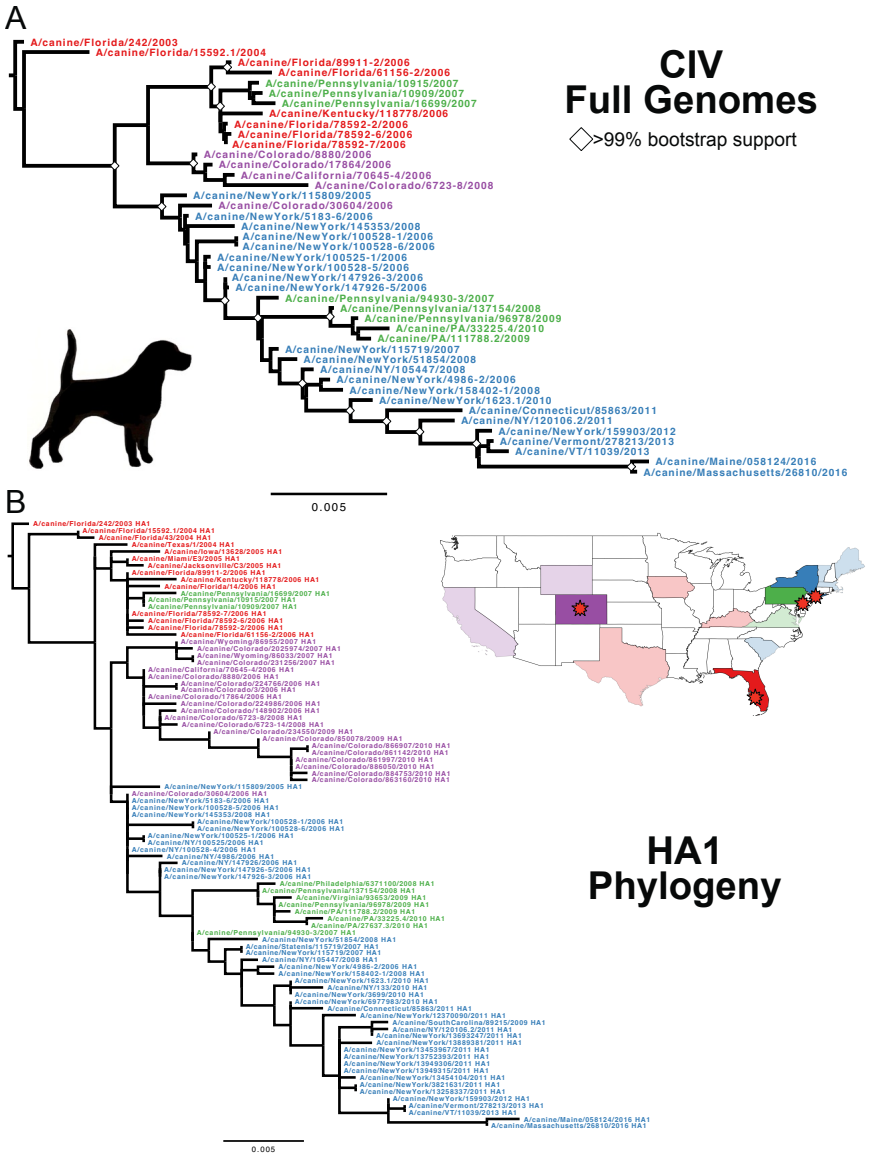

Figure 2. Evolution of the CIV H3N8 clade. (A) ML tree of the full genome data set of CIV, with taxa color defined by outbreak source region: Red, Florida/SE; Blue, New York City; Green, Philadelphia; Purple, Colorado. Tree was rooted to earliest sample, Florida/242/2003. Scale denotes nucleotide divergence.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

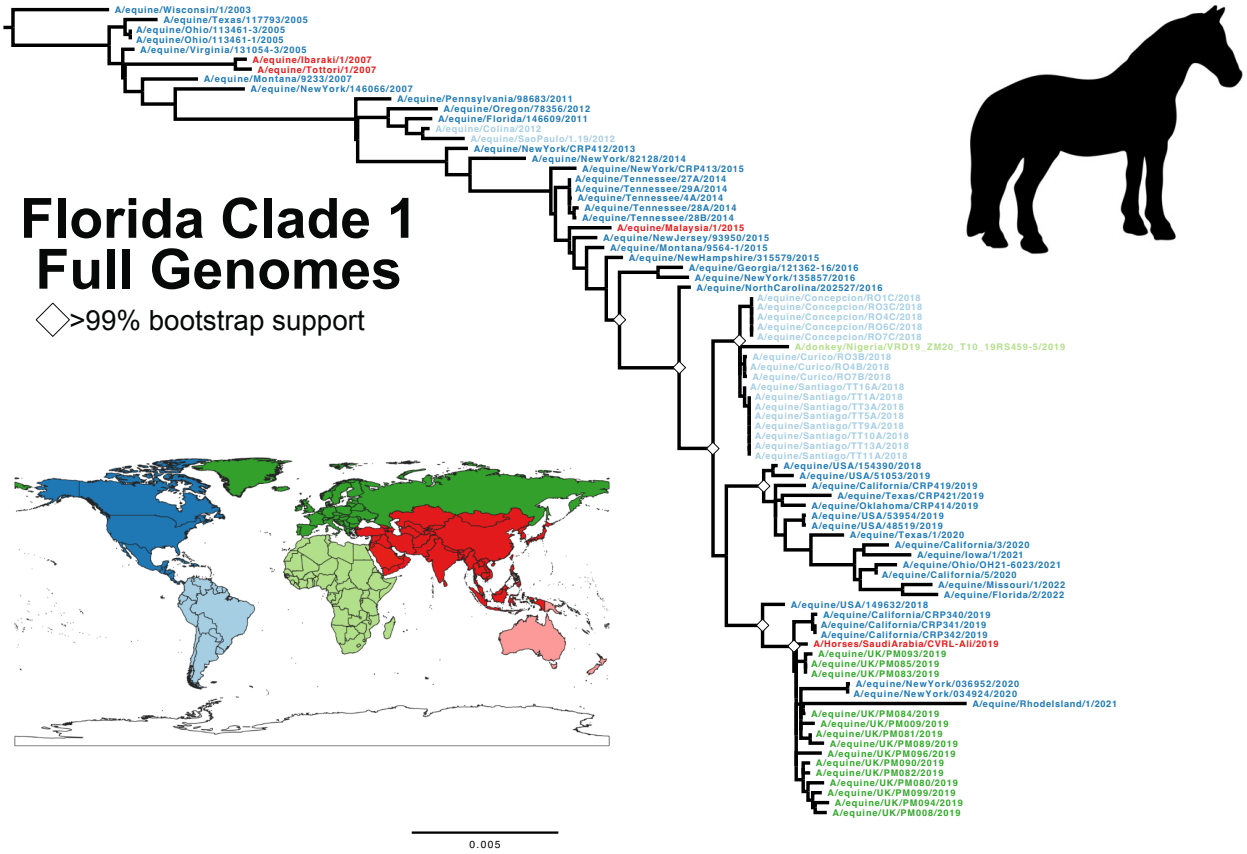

Figure 3. Evolution of the H3N8 EIV clade FC1. ML tree of full genome data set of FC1, with taxa color defined by worldwide region. Tree was rooted to earliest sample, Wisconsin/1/2003. Scale denotes nucleotide divergence. White diamonds at major nodes represent bootstrap support >99%.

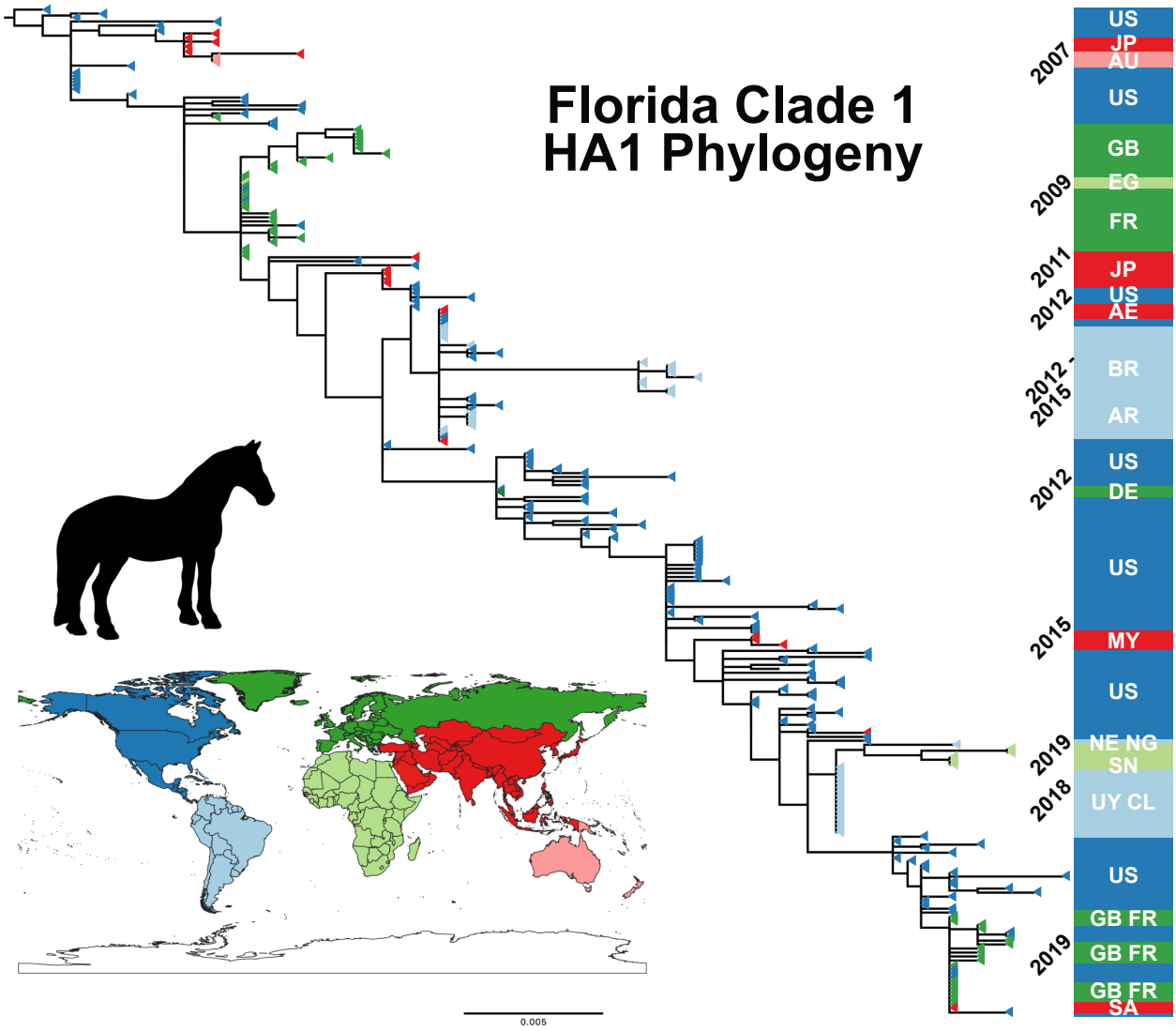

Figure 4. Evolution of the H3N8 EIV clade FC1. ML tree of the expanded HA1 data set (n=251), also colored by regional isolate. Tree was rooted to earliest sample, Wisconsin/1/2003. Scale denotes nucleotide divergence. Dates of and geography of regional subclades shows the recurring impact of US circulation 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.

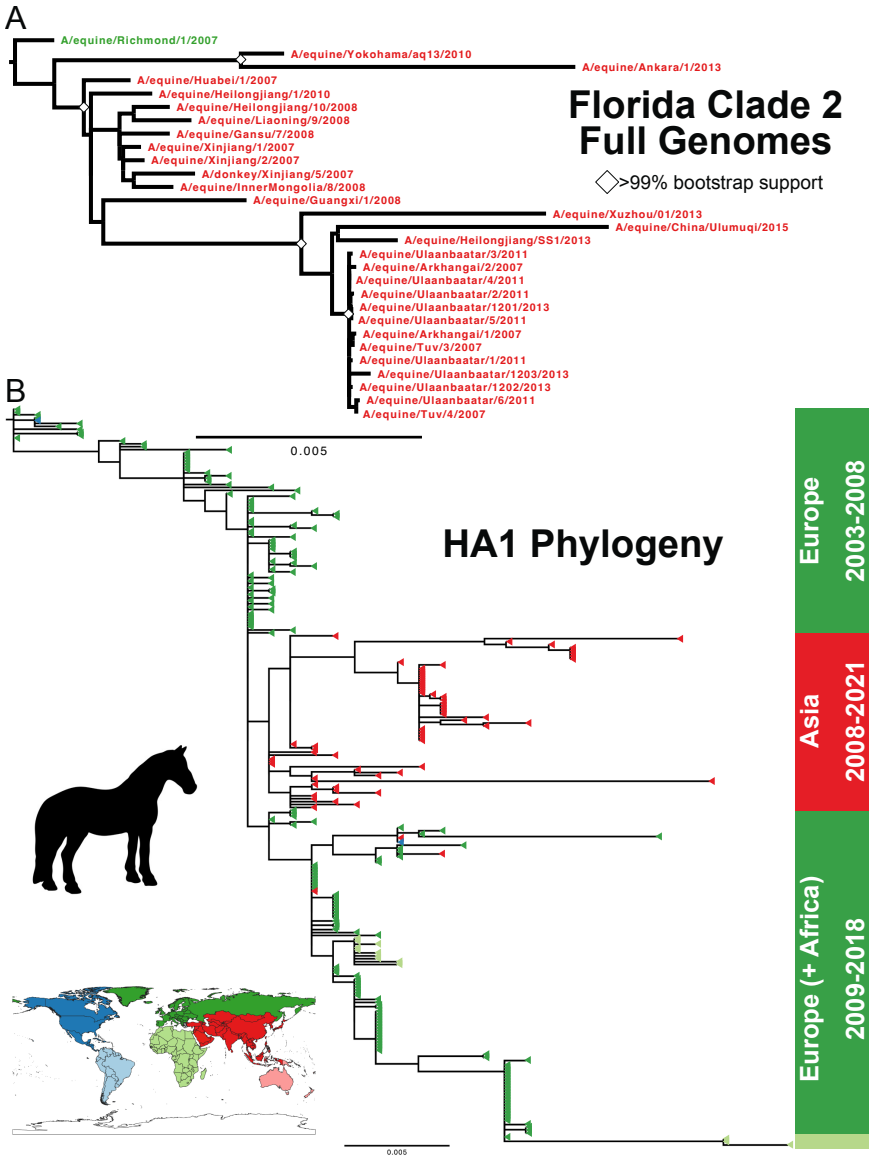

Figure 5. Evolution of the H3N8 EIV clade FC2. (A) ML tree of full genome data set of FC2, with taxa color defined by worldwide region. Tree was rooted to earliest full genome sample, Richmond/1/2007. Scale denotes nucleotide divergence. White diamonds at major nodes represent bootstrap support >99%. (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.

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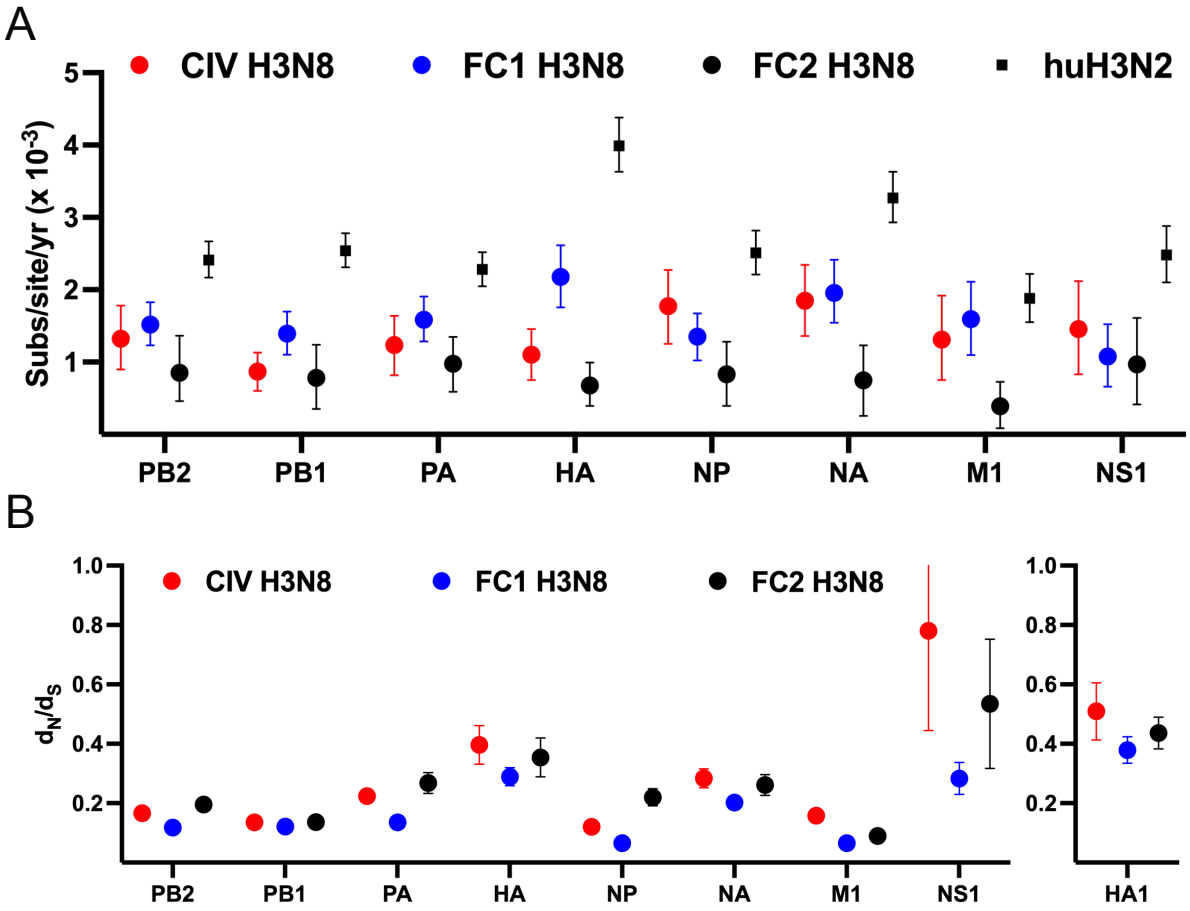

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830 **Figure 6.** Molecular evolution of the H3N8 clades. (A) The nucleotide substitution rate for each
831 segment major ORF in each clade are compared to known huH3N2 reference data set (97).
832 Datapoints are mean values and error bars represent 95% highest posterior density (HPD)
833 values. (B) The mean d_N/d_S for each segment major ORF was determined using the SLAC
834 program from the Datamonkey platform. Datapoints are mean values and error bars represent
835 95% confidence intervals (CI).

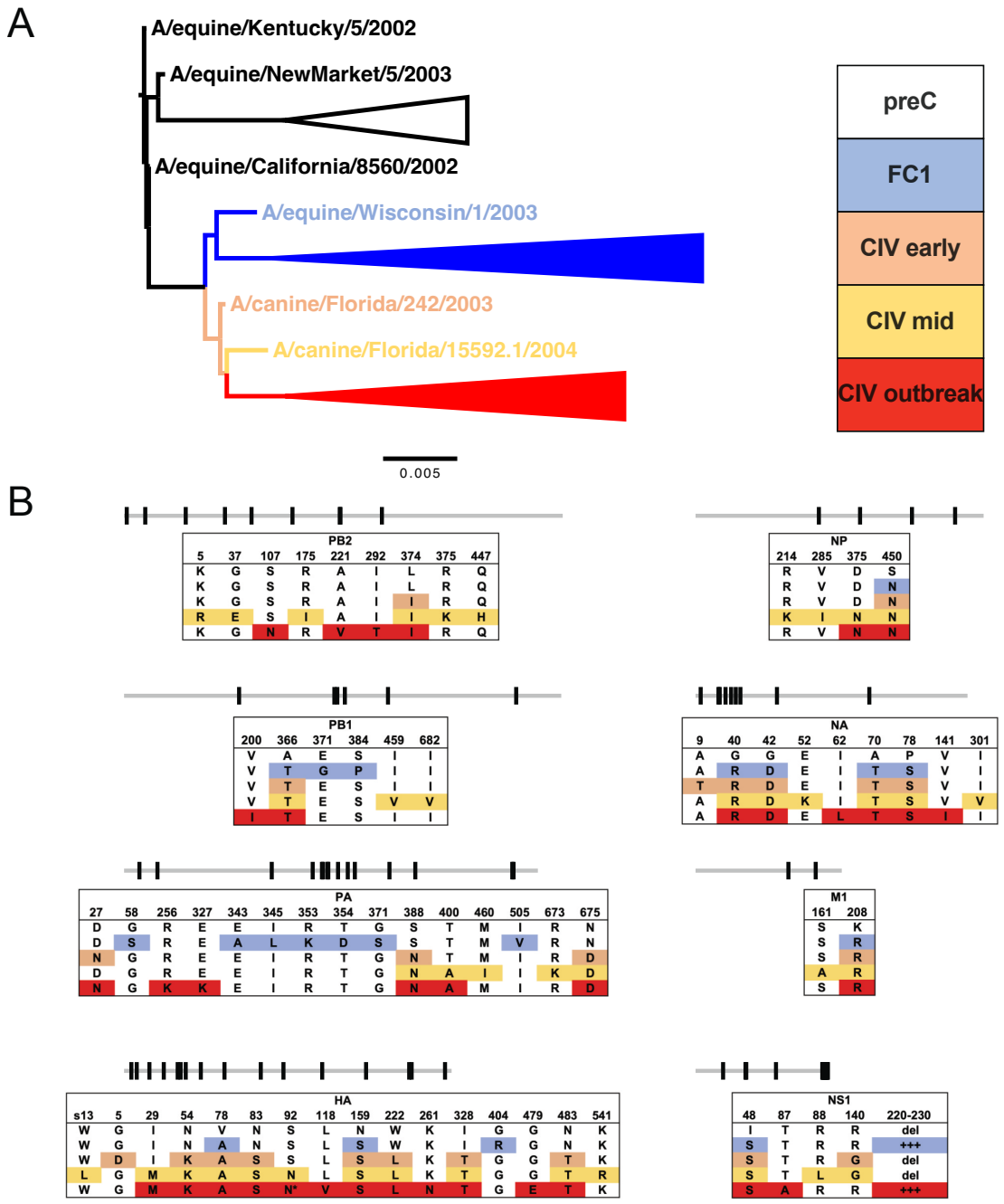

Figure 7. Early molecular evolution of H3N8 clade divergence and CIV emergence. (A) The H3N8 whole genome ML tree focused on the earliest preC, FC1, and CIV taxa for comparative alignment. (B) Key nonsynonymous changes in the earliest diverging taxa are identified by genome segment and position. Some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