1 **Understanding the Divergent Evolution and Epidemiology of H3N8**

2 **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, 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

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51   Central Asia. By comparing the hosts, epidemiology, and evolution of these influenza viruses we

52   explain how these lineages had different evolutionary fates, and show why elucidating these

53   evolutionary processes is key to understanding zoonotic disease and viral emergence. (137

54   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

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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).

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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

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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 (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

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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

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).

19

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

485

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800   **Figures and Legends**

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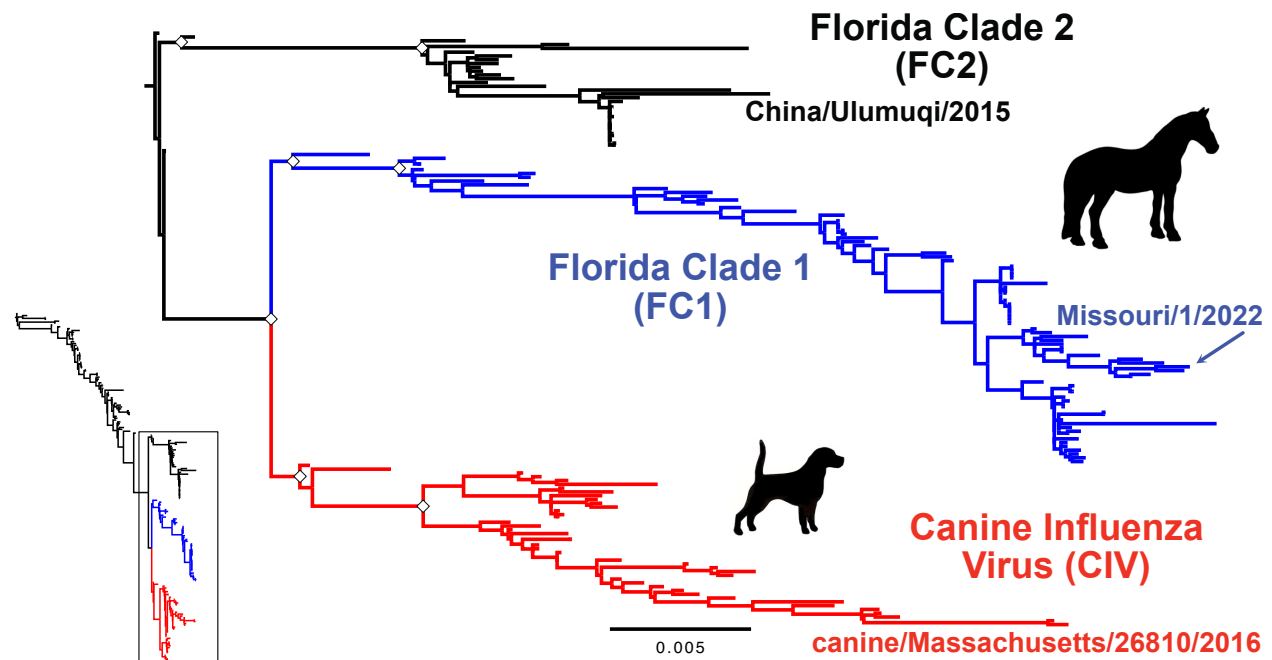
803   **Figure 1**. Recent evolution of H3N8 influenza virus. Equine influenza virus (EIV) H3N8 evolution

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.

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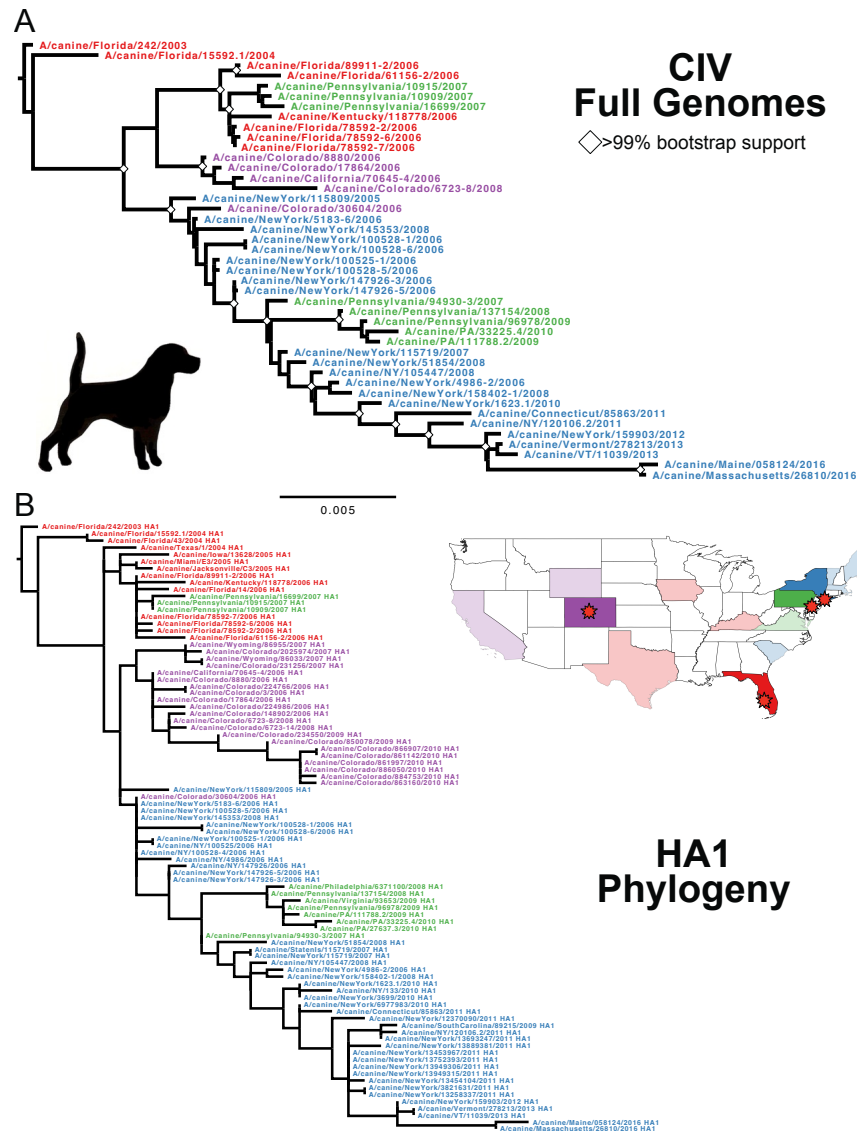
Figure 2. Evolution of the CIV H3N8 clade. (A) ML tree of the full genome data set of CIV, with

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.

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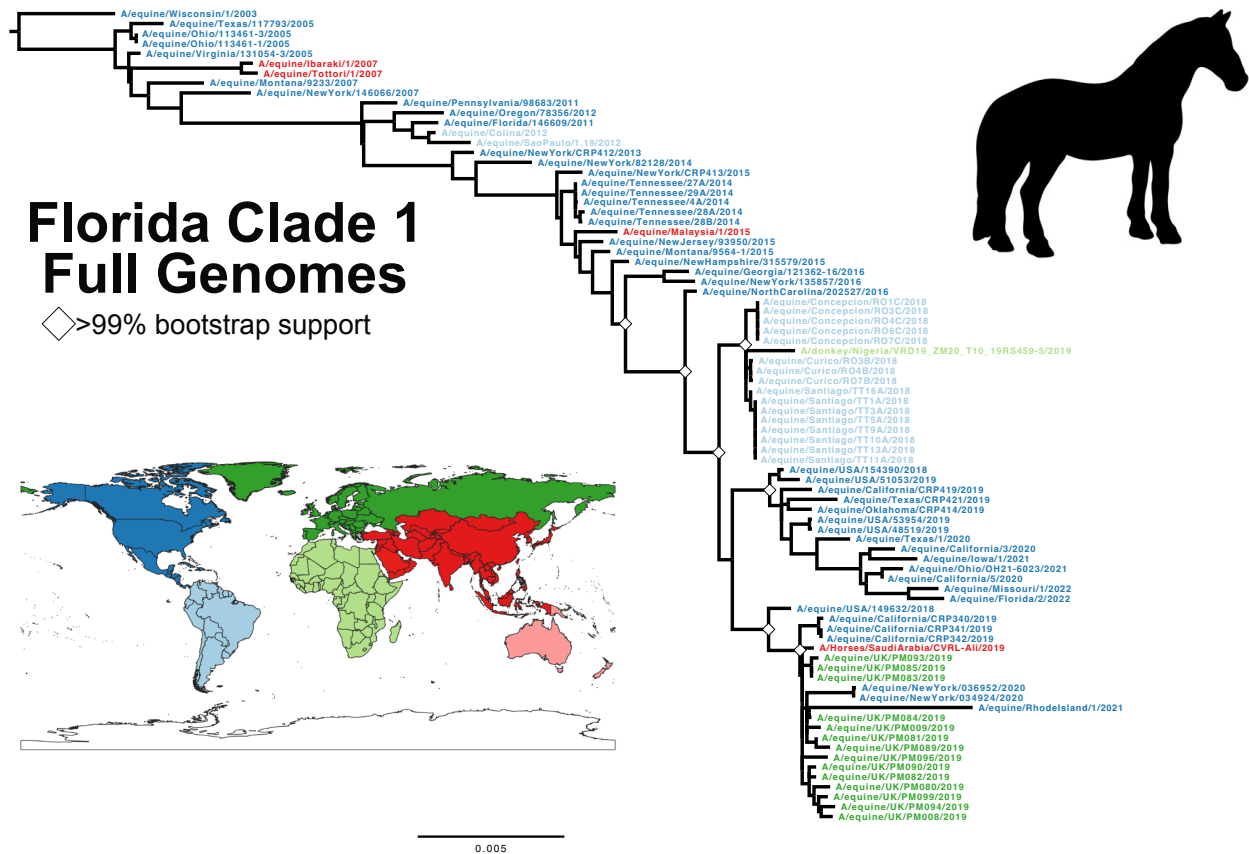
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819 **Figure 3**. Evolution of the H3N8 EIV clade FC1. ML tree of full genome data set of FC1, with

820 taxa color defined by worldwide region. Tree was rooted to earliest sample, Wisconsin/1/2003.

821 Scale denotes nucleotide divergence. White diamonds at major nodes represent bootstrap

822 support >99%.

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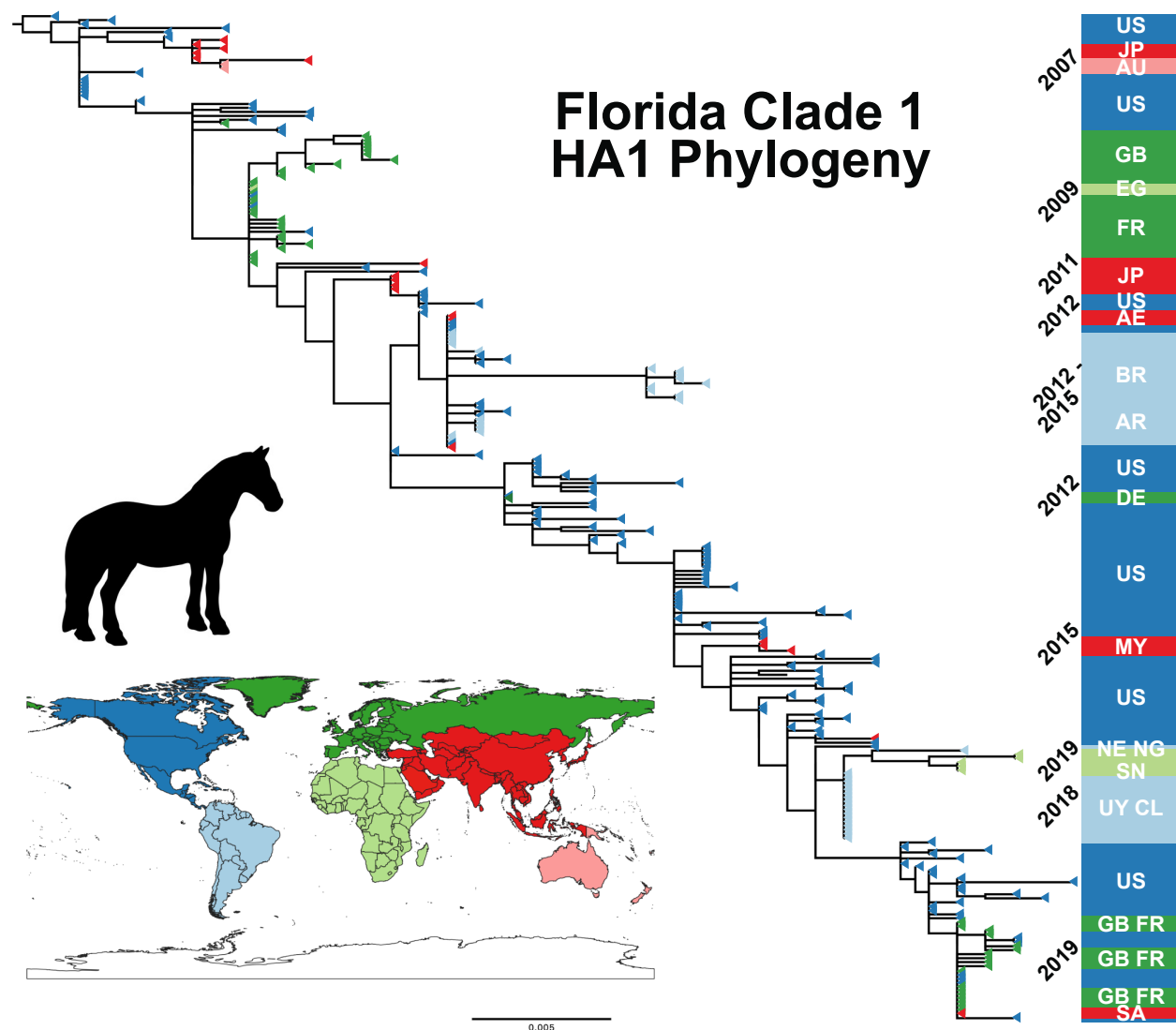
Figure 4. Evolution of the H3N8 EIV clade FC1. ML tree of the expanded HA1 data set (n=251), also colored by regional isolate. Tree was rooted to earliest sample, Wisconsin/1/2003. Scale denotes nucleotide divergence. Dates of and geography of regional subclades shows the recurring impact of US circulation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

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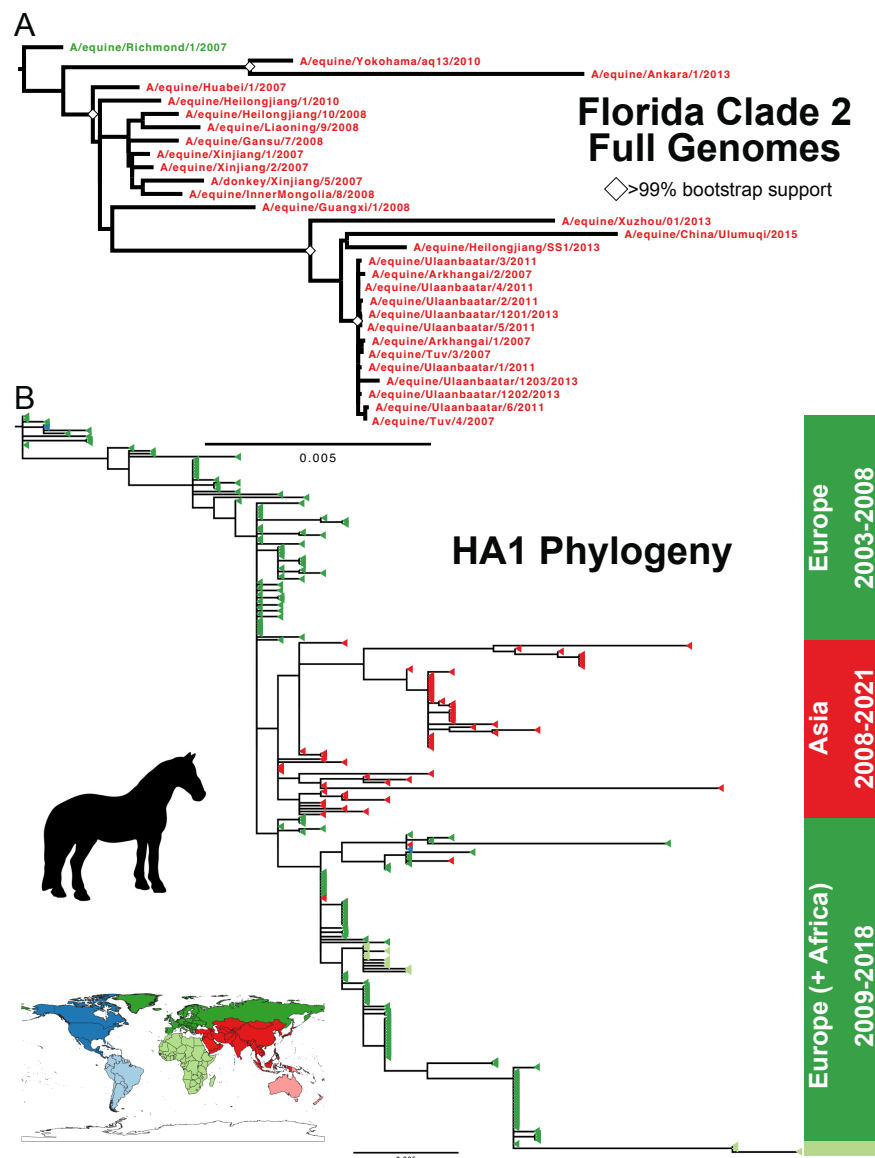
Figure 5. Evolution of the H3N8 EIV clade FC2. (A) ML tree of full genome data set of FC2, with

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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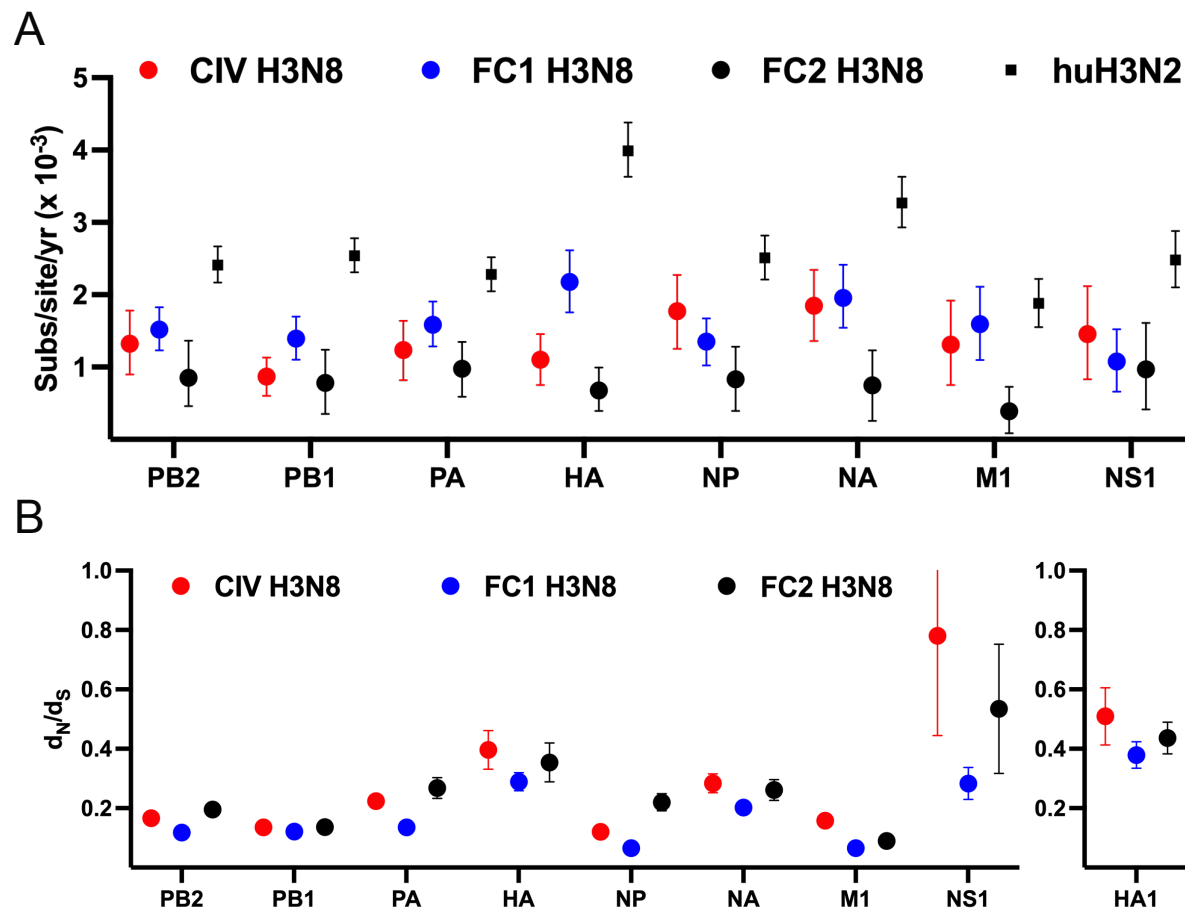
842 **Figure 6.** Molecular evolution of the H3N8 clades. (A) The nucleotide substitution rate for each

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).

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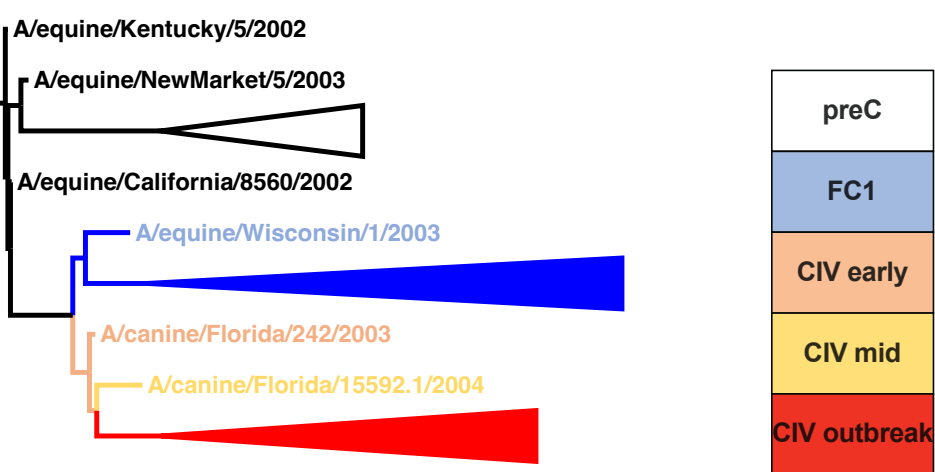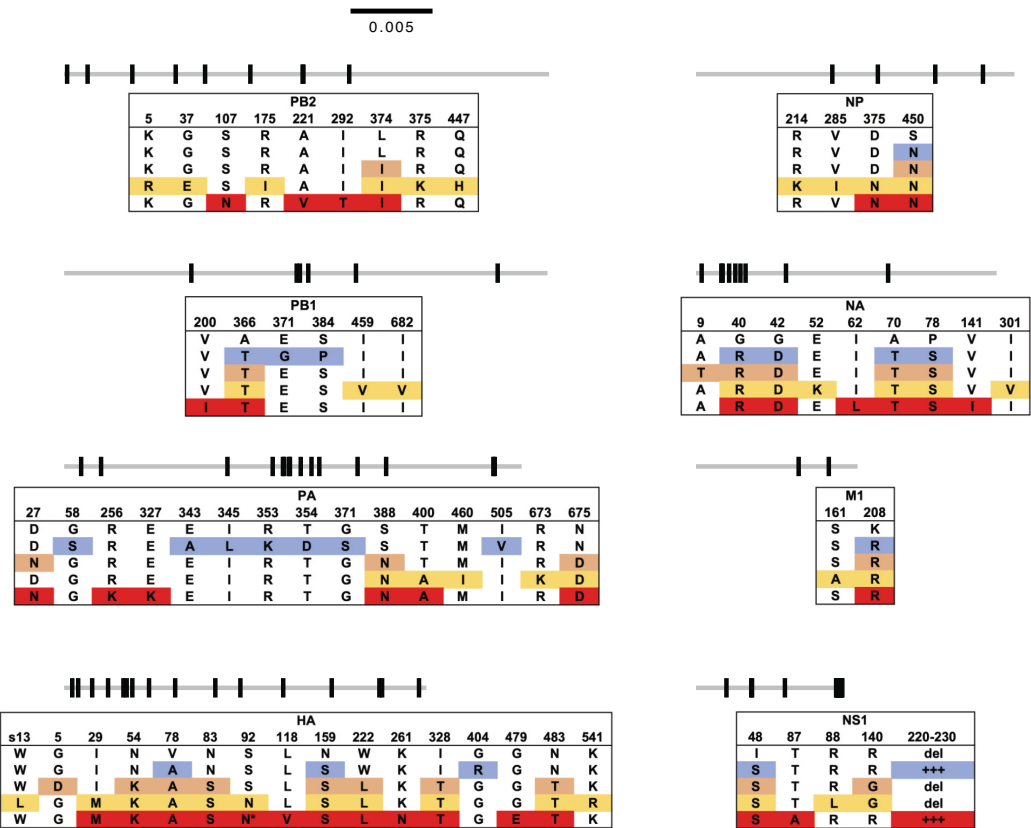
Figure 7. Early molecular evolution of H3N8 clade divergence and CIV emergence. (A) The H3N8 whole genome ML tree focused on the earliest preC, FC1, and CIV taxa for comparative alignment. (B) Key nonsynonymous changes in the earliest diverging taxa are identified by genome segment and position. Some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

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