Comparative viral metagenomics from chicken feces and farm dust in the Netherlands

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Running Head: Virome of Dutch farmed chicken feces and farm dust

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ABSTRACT (250 words, word limit = 250 words)

Livestock animals housed in close proximity to humans can act as sources or intermediate hosts facilitating animal-to-human transmission of zoonotic diseases. Understanding virus diversity in livestock is important for identifying potential zoonotic threats and for ensuring animal health and safe livestock production. Here, we report viral metagenomic characterization of chicken feces (N=51) and paired farm dust samples (N=13) using metagenomic deep sequencing. Samples were collected at 4-5 time points in three broiler farms in the Netherlands. Viruses in the *Parvoviridae* and *Picornaviridae* families were the most prevalent, detected in all feces and dust samples and in all feces and 85% of dust samples, respectively. Virus composition found in chicken feces and corresponding dust samples were similar. Great genomic diversity was identified in *Picornaviridae* and 46 sequences from five picornavirus genera (*Sicinivirus*, *Megrivirus*, *Anatvirus*, *Gallivirus* and *Avisvirus*) were detected. For calicivirus, *Basovirus* and an unclassified novel chicken calicivirus were identified in 13 fecal and 1 dust samples. Two distinct types of chicken astroviruses were identified. Phylogenetic analyses of identified virus sequences from *Picornaviridae*, *Astroviridae* and *Caliciviridae* suggested that viral sequences obtained from different farms are often more closely related to each other than global reference sequences, and sequences from feces and paired dust samples also clustered together. Importantly, our sequencing methodology enabled the recover viral genome sequences from farm dusts, allowing the tracking of virus chatter between livestock animals and their farm environment. This study, albeit relative sample size, does expand current knowledge of virus communities in chickens and surrounding dusts.

IMPORTANCE (147 words, word limit = 150 words)

Chickens may harbor various zoonotic pathogens, some of which can cause severe clinical outcomes in animals and humans. Farm dust can act as vector to facilitate zoonoses transmission. Here, we report the metagenomic characterization of virus communities of chicken feces and paired farm dust samples collected at multiple time points during the production cycle in broiler farms in the Netherlands. *Parvoviridae* and *Picornaviridae* were most frequently detected. We also identified novel astrovirus and calicivirus sequences that would inform future virus taxonomy classification. This is the first study to characterize virus communities in farmed chickens and paired farm dust samples. We also describe a dust sequencing strategy that can be adapted for future dust metagenomic characterization. Our study

30 could help setting up a surveillance baseline for tracking virus flow between chickens and their farm
31 environment which could guide zoonotic outbreak preparedness and health risk assessment of farm
32 exposure.
33

34 ## 1. INTRODUCTION

35 Most emerging infectious diseases are of zoonotic origins (1). While 71.8% of zoonoses originated
36 from wildlife, the emergence of MERS-CoV (2) and Nipah virus (3) has shown that livestock can act as
37 intermediate hosts and/or reservoirs which can sustain zoonotic transmission at the wildlife-livestock-
38 human interface. In addition to passing viruses from wildlife to humans, livestock is also reservoir of
39 zoonotic pathogens, such as bacteria *Coxiella burnetii* (*C. burnettii*) that causes Q-fever in goats, sheep
40 and cattle (4), and avian influenza (5, 6). Additionally, epidemiological studies have found evidence for
41 increased risk of respiratory disease in persons with exposure to large scale animal farming (7, 8). In part,
42 this can be attributed to farm dust exposure for which adverse health effects have been demonstrated
43 (9–11), and the potential of zoonotic infections in these cases could be important yet under-reported.
44 For instance, a Q-fever outbreak affecting approximately 4,000 people was reported in the Netherlands
45 in 2007-2010 that was linked to circulation of *C. burnettii* in local dairy goat farms and dairy sheep farms
46 (12). Q-fever was not notifiable in small ruminant farms in the Netherlands before the outbreak, making
47 early detection of zoonotic transmission of *C. burnettii* challenging (13). Similarly, MERS-CoV had been
48 circulating among dromedary camels long before it was recognized as a cause of severe respiratory
49 disease in humans (14). These livestock-associated zoonotic disease outbreaks emphasize that
50 continued surveillance of health risks due to livestock exposure is essential, especially when the
51 intensification of livestock production is widely practiced to keep up the increasing food demand for the
52 growing population (15).

53 Advances in Next-Generation Sequencing (NGS) technology have allowed simultaneous and
54 unbiased detection of all viruses and microbes in a single sample, offering great potential in catch-all
55 infectious disease surveillance. This metagenomic NGS (mNGS) approach combines the detection of
56 known target pathogens with identification of novel and/or rare viruses (16, 17) and characterization of
57 complex viral communities (18, 19). Indeed, numerous reports of virus diversity of animals including
58 bats (20–22), pigs (23–25), wild birds (26, 27), and chickens (28) have been published since the invention

59 of NGS. Our previously published systematic review of all available viral mNGS studies of common
60 livestock (29) found that metadata of available mNGS studies is often patchy and majority of the studies
61 only had a very small sample size (N ≤10). Sequencing data of most studies was also not publicly
62 available. Continued surveillance of virus communities in common livestock following the "FAIR" data
63 principle (Findable, Accessible, Interoperable and Reusable) (30) could provide a better understanding
64 of what is normal virus diversity in livestock that is an essential baseline for early detection of potential
65 zoonotic and/or animal pathogens using mNGS.

66 Chickens (*Gallus gallus domesticus*) are the most abundant domestic livestock. According to the
67 Food and Agriculture Organization of the United Nations (FAO), the global chicken population was >22
68 billion birds in 2017 (31). Chicken products include meats and eggs that are produced by broiler and
69 layer chickens respectively, and spent layer chickens can be harvested for meats as well. Chicken
70 farming ranges from backyard to large-scale commercial farms that house ten-thousands of chickens.
71 The broiler production systems usually follow a pyramidal selective breeding structure, with four
72 generations (top to bottom: great grandparents [GGPs], grandparents [GPs], parent breeders and
73 broilers) (32). Briefly, GGPs are produced by a few breeder farms and their offspring are the GPs that
74 produce parent breeders. Broilers are the final offspring that are grown for consumption. The
75 Netherlands is the second largest agricultural exporter in the world (33, 34). There are > 1 billion housed
76 chickens and 1,741 chicken farms in the Netherlands in 2020, in which one-third of them (N=637) are
77 broiler farms (35).

78 Chicken can carry various pathogens such as *Salmonella* (36), *Campylobacter* (37) and avian
79 influenza viruses including subtypes that can cause severe infection in humans like H5N1 (38) and H7N9
80 (5); and it has been found that avian influenza virus can be transmitted to humans as a result of close
81 exposure to contaminated chicken (6). Monitoring virus diversity in chickens is a key component to
82 setting the baseline for identifying potential zoonotic threats. Meanwhile, little is known about the
83 virome composition of chicken farm dust although it has been suggested that farm dust might play a
84 role in infectious disease transmission (39, 40). Farm dust consists of animal feed, bedding material,
85 animal feces, dander, mites and microorganisms (43), as well as poultry feathers in the case of poultry
86 farm dust; and farm dust exposure may cause adverse health effects (41, 42). Most studies on farm dust

87 have focused on measuring antimicrobial genes (44) and endotoxins (9, 11), yet limited has been done
88 to document the viral content of farm dust samples.
89 Here, we document metagenomic characterization of virus diversity in a comprehensive sample set
90 of chicken feces and paired farm dust samples longitudinally collected at multiple time points in three
91 commercial broiler farms across the Netherlands. We show that chicken fecal virome and farm dust
92 virome are highly comparable and stable over time, host age and farms. This study provides useful
93 insights in understanding the virus flows between chickens and poultry farm environments and would
94 contribute to the continued effort of One Health surveillance.
95

96 ## 2. RESULTS

97 *2.1 Comparative virus prevalence in chicken feces and farm dust*

98 A total of 56 pooled chicken feces and 46 individual farm dust samples were collected. Five
99 chicken fecal samples were excluded due to insufficient cDNA concentration for library preparation.
100 Individual farm dust samples collected at the same sampling moment from the same farm were pooled
101 together during sample processing for sequencing, making up a final set of 13 pooled farm dust samples.
102 Metagenomic deep sequencing of pooled chicken feces (N=51) and pooled chicken farm dust samples
103 (N=13) generated ca 1-2 million and 1-3 million reads per sample, respectively.
104 From the total of 64 chicken feces and farm dust samples analyzed, *Parvoviridae* was
105 consistently detected in all samples, followed by *Picornaviridae* with a 100% and 85% prevalence in
106 chicken feces and dust samples respectively (**Table 1**). *Astroviridae* was the third most prevalent family
107 detected in 82% chicken feces and 85% of farm dust samples, followed by *Caliciviridae* with 78%
108 (chicken feces) and 69% (farm dust samples) prevalence. The pattern of virus detection observed in dust
109 samples was similar to that in chicken feces, suggesting that dust could play a role in the virus
110 transmission of these animal viruses (**Table 1** and **Figure S1**).
111 Although the detection frequency of *Parvoviridae* was slightly higher than *Picornaviridae*, the
112 number of *Picornaviridae* contigs detected was the highest (**Figure 1**). Within the *Picornaviridae* family,
113 *Sicinivirus* was consistently detected in all chicken feces and 69% of farm dust samples (**Table 2**),
114 followed by *Anativirus* (chicken feces: 70%; farm dust samples: 61%), and *Megrivirus* (chicken feces: 43%;
115 farm dust samples: 46%). Of note, the number of contigs detected in different picornavirus genera

116 corresponded to the prevalence pattern (**Figure 2 & Figure S2**). In particular, *Anatvirus*, *Gallivirus*,
117 *Megrivirus* and *Sicinivirus* were detected in all three farms, while *Orivirus* was only identified in farm
118 F_01 and F_03 and *Avisivirus* was only found in farm F_03 (**Figure S2**). When looking at virus family level,
119 we did not observe any substantial differences in overall viral contents (**Figure 1**) and virus-specific
120 prevalence (**Figure S1**) of samples from different farms and from different production cycles (age) within
121 farms.
122
123 *2.2 Genomic diversity in viruses identified from chicken feces and farm dust*
124 2.2.1 *Picornaviridae*
125 Phylogenetic construction was performed to compare the reported *Picornaviridae* sequences
126 from 5 genera (*Avisvirus*, *Anatvirus*, *Gallivirus*, *Megrivirus* and *Sicinivirus*) with global sequences. Forty-
127 six picornavirus sequences with ≥ 80% genome coverage (chicken feces: 38; farm dust samples: 8) were
128 included in the phylogenetic analysis of the polyprotein region. The maximum-likelihood (ML)
129 phylogenetic tree (**Figure 3**) showed that the sequences identified from farm F_03, albeit small number
130 (N=12), belonged to all 5 different genera *Avisvirus*, *Anatvirus*, *Gallivirus*, *Megrivirus* and *Sicinivirus*,
131 suggesting a high viral diversity circulating among chicken in this farm. Interestingly, farm F_02 with the
132 highest number of picornavirus contigs identified (N=31) has a moderate level of viral diversity, with
133 these contigs belonging to 3 genera *Anativirus*, *Sicinivirus* and *Megrivirus*. Only 3 picornavirus genomic
134 sequences identified were from farm F_01, belonging to *Sicinivirus* (N=1) and *Anativirus* (N=2).
135 *Sicinivirus* and *Anativirus* are the most prevalent virus genera found with virus sequences detected in all
136 3 farms, while sequences of *Gallivirus* and *Avisivirus* were less prevalent, found in only 1 farm, farm
137 F_03.
138 Genome sequences detected from farm dust samples belonged to *Sicinivirus* and *Megrivirus*
139 from farm F_02 that clustered with sequences identified in chicken feces from the same farm (**Figure 3**).
140 Viruses in the *Sicinivirus* genus were detected in all chicken feces samples and 69% of dust samples with
141 the highest number of viral contigs identified in each sample as compared to other genera in the
142 *Picornaviridae* family. Slightly longer branches in the *Sicinivirus* sequences in the ML tree indicates
143 higher diversity within this *Sicinivirus* clade (**Figure 3)**. We further reconstructed a ML tree for all
144 identified *Sicinivirus* polyprotein nucleotide sequences (N=15; chicken feces: 11; farm dust samples: 4) in

145 this study and compared them with global reference sequences (**Figure 4**). Two sequences from chicken
146 feces (V_M_005_picorna_5 from farm F_01 and V_M_034_picorna_3 from farm F_03) were most
147 distinct from the rest of the identified sequences (sharing only 69.4-72.3% nt identity) and were most
148 closely related to strain JSY previously identified in China (78.9-79.0% nt identity). Other *Sicinivirus*
149 sequences from the same farm often formed a monophyletic lineage, suggesting within-farm *Sicinivirus*
150 sequences are highly genetically similar. Viruses identified from chicken feces and farm dust samples
151 consistently clustered with each other forming their own sub-clusters within major clades in the ML
152 trees, indicating high similarity between sequences from feces and dusts and that they are more closely
153 related as compared to global reference sequences.
154
155 2.2.2 *Astroviridae*
156 Sixteen astrovirus sequences with ≥ 80% genome coverage were identified from this study,
157 among which only 1 astrovirus sequence was found in farm dust (VE_7_astro_14; farm F_02). There was
158 only 1 astrovirus genomic sequence from farm F_01. RNA-dependent RNA polymerase (RdRp) and
159 capsid regions of these sequences were extracted for phylogenetic analyses (**Figure 5A and 5B**,
160 respectively). ML trees of both regions showed that these 16 sequences belonged to two distinct
161 lineages, A1 and A2 (**Figure 5**). Sequences from farm F_02 and F_03 were found in both lineages A1 and
162 A2, suggesting co-circulation of different astrovirus strains in both farms. The 12 sequences from lineage
163 A1 were most closely related to avian nephritis virus strains previously reported in China and Brazil,
164 while the 4 sequences from lineage A2 were more closely related to each other and clustered with
165 chicken astroviruses previously identified in Malaysia, Canada and the USA. The 12 sequences from
166 lineage A1 shared 96-100% amino acid (aa) identity in the RdRp region while 4 sequences from lineage
167 A2 shared a higher 99-100% aa identity. For the less conserved capsid region, three small sub-lineages
168 can be observed within lineage A1 (**Figure 5B**). Amino acid identity matrix also supported this
169 observation that intra-sub-lineage identity (range 87%-100%) is higher than inter-sub-lineage identity
170 (range 64-74%). Notably, the only astrovirus sequence found in dust (strain VE_7_astro_14) was
171 identical to astrovirus recovered from its paired chicken feces sample (strain V_M_038_astro_4) at the
172 same farm.
173

174 2.2.3 *Caliciviridae*

175 Similar to astrovirus distribution in feces versus dusts, 13 out of 14 calicivirus sequences (≥ 80%
176 genome coverage) identified were recovered from fecal samples and only 1 calicivirus sequence
177 identified from dust sample. Partial ORF1 polyprotein region of these sequences was extracted to
178 construct ML tree comparing the local calicivirus with global sequences. The ML tree showed that the
179 reported calicivirus sequences formed two distinct lineages (C1 and C2) (**Figure 6**). All calicivirus
180 sequences from farm F_02 and F_03 belonged to lineage C1, while sequences from F_01 belonged to
181 both lineages C1 and C2 suggesting higher diversity of calicivirus circulating in farm F_01. The 11
182 sequences in lineage C1 shared 98.7-100% aa identity despite being collected from different farms and
183 they shared 97.2-99.1% aa similarity with two partial ORF1 sequences identified in Germany in 2010
184 (F10026n and F10034n). BLAST searches and multiple sequence alignment indicated that these 11 local
185 calicivirus sequences are the first report of complete ORF1 sequence of that particular chicken calicivirus
186 lineage. The only calicivirus sequence from dust (VE_8_calici_66) clustered in lineage C1. The 3
187 sequences in lineage C2 are highly similar, sharing 99.9-100% aa identity and were all collected from
188 farm F_01 at two time points. These 3 sequences shared 93.8-96.8% aa similarity with reference
189 sequences from USA, Korea, Brazil and Germany (*Bavaria virus*, *Bavovirus* genus*)*.
190

191 2.2.4 *Coronaviridae*

192 Three nearly-complete coronavirus genome sequences were identified in 3 chicken feces (V_M_001,
193 V_M_002 and V_M_003) from farm F_01. BLAST analyses showed that these sequences are Infectious
194 bronchitis virus (IBV) strains and share 99.92-99.95% identity at the nucleotide level with IBV vaccine
195 strain 4/91 (GenBank accession number KF377577), suggesting these IBV may possibly be viral shedding
196 following vaccination or environmental contamination as vaccines were given by drinking water.
197

198 3. DISCUSSION

199 Livestock kept in close proximity to humans can act as intermediate hosts and/or sources to amplify
200 zoonotic disease transmission (45). The surge of zoonotic disease outbreaks around the world has
201 prompted the need for more proactive surveillance strategy involving animal surveillance, which could
202 potentially guide zoonotic outbreak preparedness. Chicken is the most abundant livestock in the world

203 (31) and has been identified as reservoir for various zoonotic pathogens (37, 38). In this study, we
204 employed viral metagenomic deep sequencing to document total viruses present in chicken feces and
205 paired farm dust, data from which provide insights into virus flow between chickens and their farm
206 environment as well as a platform for future surveillance in farm animals and environmental samples.
207 To our knowledge, this is the first study characterizing the virome (viral metagenomics) of chicken feces
208 and farm dust from the Netherlands.

209 In this study, we described a primer-independent viral metagenomic deep sequencing strategy to
210 sequence viruses in dust samples that usually possess low viral load and are hence difficult to sequence.
211 We further demonstrated that using this method, we were able to recover long viral sequences with
212 ≥80% genome coverage from farm dust samples. This method could be adapted for future metagenomic
213 characterization of virus diversity in dust samples. Farm dust is associated with adverse respiratory
214 effects observed in farm workers with prolonged exposure (41). There is a knowledge gap in virus
215 diversity of farm dust. Characterizing farm dust virome could aid in investigating possible health effects
216 of occupational and environmental exposure to virus-containing farm dust.

217 Phylogenetic analyses indicated that viruses identified from farm dust samples were genetically
218 closely related to viruses identified from chicken feces collected from the same farm, suggesting that
219 viruses identified in dust were attributed to the viruses circulating in the chicken feces that is also
220 source of farm dust. This observation is in agreement with a previous study that reported the
221 correlation found in the resistomes between animal feces and farm dust (46). More importantly, the
222 detection of chicken-related virus sequences in dust samples raises the possibility of dust-borne
223 transmission of viruses especially important in the cases of chicken and/or zoonotic pathogenic viruses
224 (47), although infectivity of the viruses identified was not confirmed as no virus culture was performed
225 in our study. Future investigation on the infectivity of viruses in farm dust could provide useful insights
226 in mapping virus flows between and among chickens as well as chicken and surround environment
227 including dust, which collectively would guide future outbreak control strategies in farms.

228 Viruses identified in chicken feces from different farms are phylogenetically closely related to each
229 other and formed sub-lineages or monophyletic lineages when comparing to global related sequences.
230 This could possibly be explained by the pyramid structure of broiler production system which chicks
231 were bought from a few suppliers that were not bred by the broiler farmers; in other words, chicks

232 carrying similar viruses from a supplier were then distributed to different broiler farmers, and further
233 spread within the farms, leading to similar viruses circulating in different broiler farms. Of note, the
234 pyramid production structure was also suggested as a possible transmission routes for ESBL/pAmpC
235 producing bacteria between broiler farms (32). Future characterization of virus diversity of chickens
236 from the top part of the pyramid is needed to confirm the speculation.

237 Viruses belonging to five genera of the *Picornaviridae* family were identified in our study, suggesting
238 a great diversity of picornaviruses circulating in chickens, which is consistent with previous studies that
239 detected various picornavirus genera in chicken respiratory samples (48, 49). Astroviruses identified in
240 this study clustered in two distinct phylogenetic lineages; these astroviruses could present possible new
241 genotypes. For astrovirus, all chicken astroviruses are grouped to one single species according to the
242 current taxonomy assignment from International Committee on Taxonomy of Viruses (50). Current
243 classification of avastrovirus is based on phylogenetic analysis of capsid (ORF2) amino acid sequences
244 (51). More comprehensive assignment of genotypes is hindered by limited number of complete capsid
245 sequences. A similar observation was seen in *Caliciviridae*, we identified two types of caliciviruses
246 forming two distinct lineages in the ML tree. Lineage C2 is closely related to the *Bavovirus* genus, while
247 the sequences from C1 are most related to an unclassified chicken calicivirus strain F10026n (52) that
248 only possessed a partial ORF1 sequence. Our data have provided 16 astrovirus and 14 calicivirus
249 sequences, which help expand the current knowledge of chicken astrovirus and calicivirus diversity and
250 could potentially contribute to improve future taxonomy assignment.

251 We observed that viruses in the *Parvoviridae* and *Picornaviridae* families were consistently detected
252 in chicken feces and farm dust samples at multiple time points in all three farms. These virus families
253 could potentially present the "signature" viruses to indicate chicken exposure. By combining these
254 viruses together, a chicken viral fingerprint profile may be built that can be used to map the virus flow
255 between farms and surrounding areas. In case of any future avian-associated zoonotic outbreaks, these
256 marker viruses could also be used to assess spatial transmission risk in poultry farm surrounding areas
257 (29, 53). Additionally, these viruses could also be used as markers to assess the efficiency of disinfection
258 procedures in farms and surrounding environments although stabilities of different viruses may differ
259 due to different structures. Since this is the first study of its kind in chicken and farm dust in the
260 Netherlands, further and perhaps larger scale of surveillance with the inclusion of samples from layer

261 hens and more environmental samples from surrounding areas are certainly necessary to confirm our
262 observation and the applicability of using these identified viruses as signature virus fingerprint.
263 In conclusion, we provide a comprehensive dataset of virus diversity of chicken feces and paired
264 farm dust samples. Highly prevalent viruses like parvoviruses and picornaviruses were detected and
265 they could potentially serve as marker viruses to follow the virus flows between chickens and their
266 surrounding environment. Identification of novel calicivirus and astrovirus sequences indicates that
267 continued effort is needed for increasing understanding of virus diversity in chicken feces. Viral
268 surveillance using the One Health approach is warranted for early detection of zoonotic viruses. In the
269 long term, understanding virus flows at the animal-human-environmental interface is vital for
270 identifying potential zoonotic threats and zoonotic outbreak control and preparedness.
271

272 ## 4. MATERIALS AND METHODS

273 *4.1 Sample collection*

274 Pooled chicken feces and farm dust samples were collected in three commercial broiler farms in the
275 Netherlands at 4-5 time points from May to August 2019. The three commercial broiler farms are
276 located in three different regions in the Netherlands (Western part: Noord/Zuid-Holland region;
277 Northern part: Friesland/Groningen region; Eastern part: Gelderland region). Detailed sampling strategy
278 and sample metadata is described in **Table 3**. Each pooled poultry fecal sample contains fresh fecal
279 material from 3-4 chicks. Farm dust samples were collected using a passive air sampling approach using
280 electrostatic dustfall collectors (EDCs) (54, 55). Electrostatic cloths were sterilized through overnight
281 incubation at 200°C. Sterilized electrostatic cloths were then fixed to a pre-cleaned plastic frame. EDCs
282 were exposed for 7 days at 1 meter above the floor with the electrostatic cloths facing up in broiler
283 farms to enable sampling of settling airborne dust instead of resuspended dust from the floor. EDCs
284 were contained in a sterile plastic bag before and after sampling. All samples were transported under
285 cold chain management and stored at -20°C/-80°C before processing.
286

287 *4.2 Sample processing and metagenomic deep sequencing*

288 Chicken fecal samples were processed as previously described (56). Briefly, chicken fecal suspension
289 was prepared in Phosphate-buffered saline (PBS) and treated with TURBO DNAse (Thermo Fisher, USA)

290 at 37 °C for 30 minutes, and then subjected to total nucleic acid extraction using QIAamp viral RNA mini
291 kit (Qiagen, Germany) according to manufacturer's instruction without addition of carrier RNA. For dust
292 samples, electrostatic cloths were incubated in 3% beef extract buffer for 1 hour on rolling as previously
293 published (57). After incubation, the suspension was collected and centrifugated at 4,000 g at 4°C for 4
294 minutes to pellet any large particles or debris. Total viruses in dust suspension were concentrated using
295 polyethylene glycol (PEG) similar to virus concentration in sewage published previously (58, 59). Briefly,
296 PEG 6000 was added to each dust suspension to make up a final 10% PEG 6000 (Sigma-Aldrich, USA)
297 concentration, followed by pH adjustment to pH 4 and overnight incubation at 4°C with shaking. After
298 overnight incubation, sample was centrifuged at 13,500 g at 4°C for 90 minutes. Supernatant was
299 removed and the remaining pellet was resuspended in 500 μL of pre-warmed glycine buffer, and then
300 subjected to 5-minute centrifugation at 13,000 g at 4°C. Supernatant was collected, and supernatants
301 from EDC samples that were collected in the same farm at the same time point (N=1-4) were pooled
302 together for further processing. Viral-enriched dust suspension samples were then treated with TURBO
303 DNase as previously described (56) to remove non-encapsulated DNA, followed by total nucleic acid
304 extraction using MagMAX™ viral RNA isolation kit (Thermo Fisher, USA) according to manufacturer'
305 instructions but without the use of carrier RNA. Reverse transcription and second strand cDNA synthesis
306 of chicken fecal samples and dust samples was performed as previously described (56), using
307 SuperScript III reverse transcriptase and non-ribosomal hexamers (60) (reverse transcription) and
308 Klenow fragment 3'-5' exo- (New England BioLabs, USA) (2nd strand cDNA synthesis), respectively.
309 Standard Illumina libraries were prepared using Nextera XT DNA library kit following the
310 manufacturer's instruction. Final libraries were sequenced on the Illumina MiSeq platform (600 cycles;
311 paired-end). Chicken fecal samples with a multiplex range of 16-19 samples. Dust samples and negative
312 blank controls were sequenced with a multiplex of 12 samples per run.
313
314 *4.3 Sequencing data analysis*
315 Raw reads were subjected to adapter removal using Trim Galore/default Illumina software, followed
316 by quality trimming using QUASR (61) with a threshold of minimum length of 125 nt and median Phred
317 score ≤ 30. Remaining reads were *de novo* assembled using metaSPAdes v3.12.0 (62). *De novo*
318 assembled contigs were classified using UBLAST (63) against eukaryotic virus family protein databases as

319 previously described (22, 25). We set a detection threshold of contig with minimum amino acid identity
320 of 70%, minimum length of 300 nt and an e-value threshold of 1 x 10^{-10} when interpreting our contig
321 classification results. Contig classification results were analyzed and visualized using R packages
322 including dplyr, reshaped2 and ComplexHeatmap (64–66).
323
324 *4.4 Phylogenetic analysis*
325 Sequences with ≥ 80% of genome coverage from the *Picornaviridae*, *Astroviridae* and *Caliciviridae*
326 families were used for phylogenetic analyses, comparing them with global sequences retrieved from
327 GenBank. Multiple sequence alignment was performed using MAFFT (67) and manually checked in
328 Geneious v2021.0.3 (Biomatters, New Zealand). Best-fit models of evolution for phylogenetic tree
329 reconstruction were estimated using ModelFinder module in IQ-TREE v1.6.11 (68), determined by
330 Akaike Information Criterion (AIC). Maximum-likelihood trees were constructed using RAxML-NG v1.0.1
331 (69) with 100 pseudo-replicates. Resulting trees were visualized and annotated using R package ggtree
332 (70).
333
334 *4.5 Data availability*
335 The raw reads are available in the SRA under the BioProject accession number PRJNA670873 (chicken
336 feces) and PRJNA701384 (farm dust samples) (**Table S1**). All sequences in phylogenetic analysis have
337 been deposited in GenBank under the accession numbers MW684778 to MW684847 (**Table S2**).
338
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346
347

348 **AUTHOR CONTRIBUTION**

349 K.T.T.K and M.V.T.P conceived the study and performed experiments. M.M.T.dR and I.M.W. designed
350 sampling strategy. M.M.T.dR. and A.B.M. handled sample collection and collected associated metadata.
351 K.T.T.K. and M.V.T.P. analyzed data. K.T.T.K. drafted the manuscript. M.V.T.P., M.P.G.K., L.A.M.S., I.M.W.,
352 M.M.T.dR and A.B.M. edited the manuscript. M.V.T.P., M.P.G.K. and I.M.W. supervised the study.
353 M.P.G.K., D.J.J.H. and L.A.M.S. acquired the funding.
354

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Figure legends

Figure 1. An overview of virus contigs detected in chicken feces and chicken farm dust samples. Each column represents one sample. Associated metadata is shown at the top panel. The color intensity of the heatmap (bottom panel) is determined by number of contigs with minimum length of 300 nt/bp, at least 70% identity and an e-value threshold of $1x10^{-10}$ when comparing with closest reference in our database. Sample order is assorted by farm ID, sampling time point. Time point is an arbitrary number. Samples were collected at the same time and place when both Farm ID and Time point match.

Figure 2. An overview of picornavirus contigs detected in chicken feces and chicken farm dust samples. Each column represents one sample. Associated metadata is shown at the top panel. The color intensity of the heatmap (bottom panel) is determined by number of contigs with minimum length of 300 nt, at least 70% identity and an e-value threshold of $1x10^{-10}$ when comparing with closest reference in our database. Sample order is assorted by farm ID, sampling time point. Time point is an arbitrary number. Samples were collected at the same time and place when both Farm ID and Time point match.

Figure 3. Maximum-likelihood phylogenetic tree of 76 (sequences from this study: 46; reference sequences: 30) picornavirus partial polyprotein amino acid sequences (3786 sites) reconstructed using a best-fit evolutionary model LG+FC+R10. For clarity, only bootstrap supports >= 70 are shown as a navy-blue node point. Tip labels of our samples are colour-coded by corresponding farm ID. Reference sequences are in grey. Tree was mid-point rooted for clarity.

Figure 4. Maximum-likelihood phylogenetic tree of 30 sicinivirus partial polyprotein nucleotide sequences (sequences from this study: 15; reference sequences: 15; 8672 sites) reconstructed using a best-fit evolutionary model GTR+F+R10. For clarity, only bootstrap supports >= 70 are shown as a navy-blue node point. Tip labels of our samples are colour-coded by corresponding farm ID. Reference sequences are in grey and tree was mid-point rooted for clarity.

Figure 5. A. Maximum-likelihood phylogenetic tree of 33 astrovirus partial RdRp amino acid sequences (sequences from this study: 16; reference sequences: 17; 548 sites) reconstructed using a best-fit evolutionary model LG+FC+I+G4m. B. Maximum-likelihood phylogenetic tree of 33 astrovirus partial capsid amino acid sequences (sequences from this study: 16; reference sequences: 17; 908 sites) reconstructed using a best-fit evolutionary model LG+FC+I+G4m. For clarity, only bootstrap supports >= 70 are shown as a navy-blue node point. Tip labels of our samples are colour-coded by corresponding farm ID. Reference sequences are in grey and tree was mid-point rooted for clarity.

Figure 6. Maximum-likelihood phylogenetic tree of 30 calicivirus partial polyprotein amino acid sequences (sequences from this study: 14; reference sequences: 16; 2794 sites) reconstructed using a best-fit evolutionary model LG+FC+R10. For clarity, only bootstrap supports >= 70 are shown as a navy-blue node point. Tip labels of our samples are colour-coded by corresponding farm ID. Reference sequences are in grey and tree was mid-point rooted for clarity.

Table 1. Prevalence of different eukaryotic virus families in chicken feces and farm dust samples

Sample types		Chicken feces (N=51)	Farm dust (N=13)
Virus family	***Astroviridae***	42 (82%)	11 (85%)
	Caliciviridae	40 (78%)	9 (69%)
	Coronaviridae	19 (37%)	3 (23%)
	Parvoviridae	51 (100%)	13 (100%)
	Picobirnaviridae	8 (16%)	2 (15%)
	Picornaviridae	51 (100%)	11 (85%)
	Reoviridae	7 (14%)	3 (23%)

Table 2. Prevalence of picornavirus genera in chicken feces and farm dust samples

Sample types		Chicken feces (N=51)	Farm dust (N=13)
Picornavirus genera	***Anativirus***	36 (70%)	8 (61%)
	Avisivirus	3 (5%)	0 (0%)
	Gallivirus	16 (31%)	4 (30%)
	Megrivirus	22 (43%)	6 (46%)
	Orivirus	8 (15%)	0 (0%)
	Sicinivirus	51 (100%)	9 (69%)

Table 3. Detailed sampling strategy

Farm #	Sampling moment identifier	Time point	No. of pooled fecal sample(s) collected	No. of pooled EDC sample(s) collected (no. of individual EDC sample[s] collected)	Week production cycle
F_01	F_01_1	1	4	1 (4)	3
	F_01_2	2	4	1 (4)	4
	F_01_3	3	4	1 (4)	5
	F_01_4	4	4	1 (1)	4
	F_01_5	5	4	0 (0)	5
F_02	F_02_1	1	4	1 (2)	4
	F_02_2	2	4	1 (4)	5
	F_02_3	3	4	1 (4)	2
	F_02_4	4	4	1 (4)	4
	F_02_5	5	4	1 (4)	5
F_03	F_03_1	1	4	1 (3)	4
	F_03_2	2	4	1 (4)	5
	F_03_3	3	4	1 (4)	4
	F_03_4	4	4	1 (4)	5

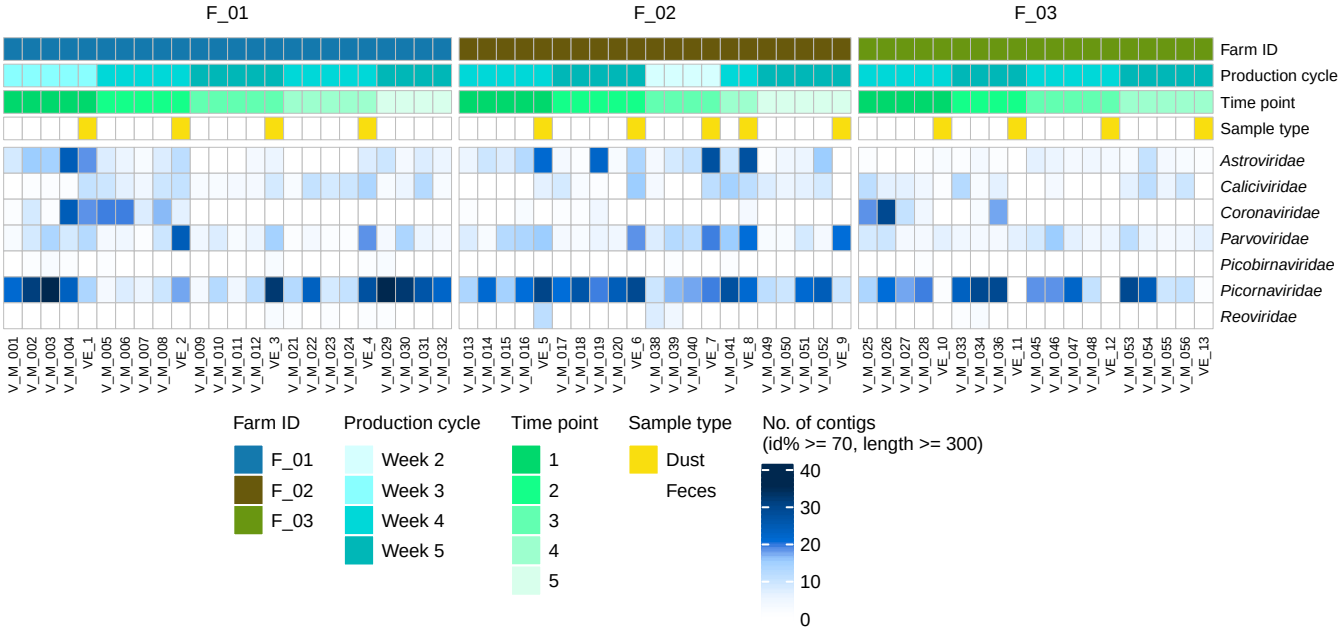
Supplementary materials legends.

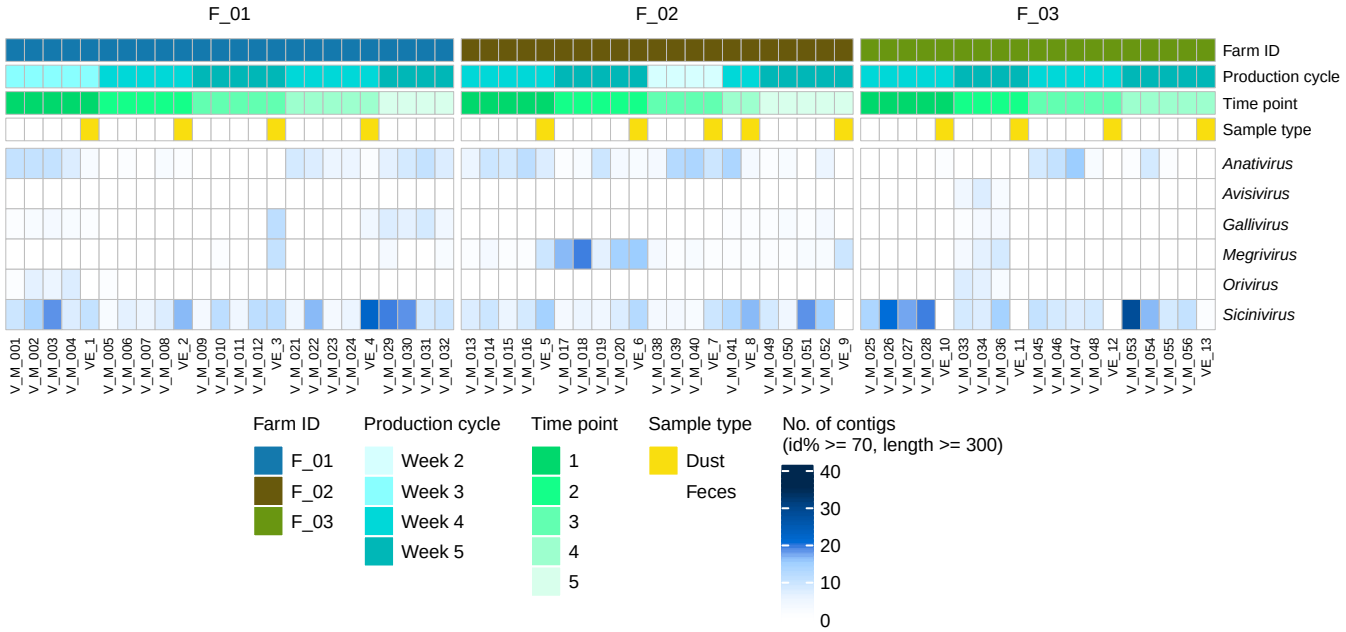
Figure S1. An overview of virus family prevalence in chicken feces and farm dust samples. Associated metadata is shown at the top panel. The color intensity of the heatmap (bottom panel) is determined by number of contigs with minimum length of 300 nt, at least 70% identity and an e-value threshold of $1x10^{-10}$ when comparing with closest reference in our database. Sample order is assorted by farm ID, sampling time point. Time point is an arbitrary number. Samples were collected at the same time and place when both Farm ID and Time point match.

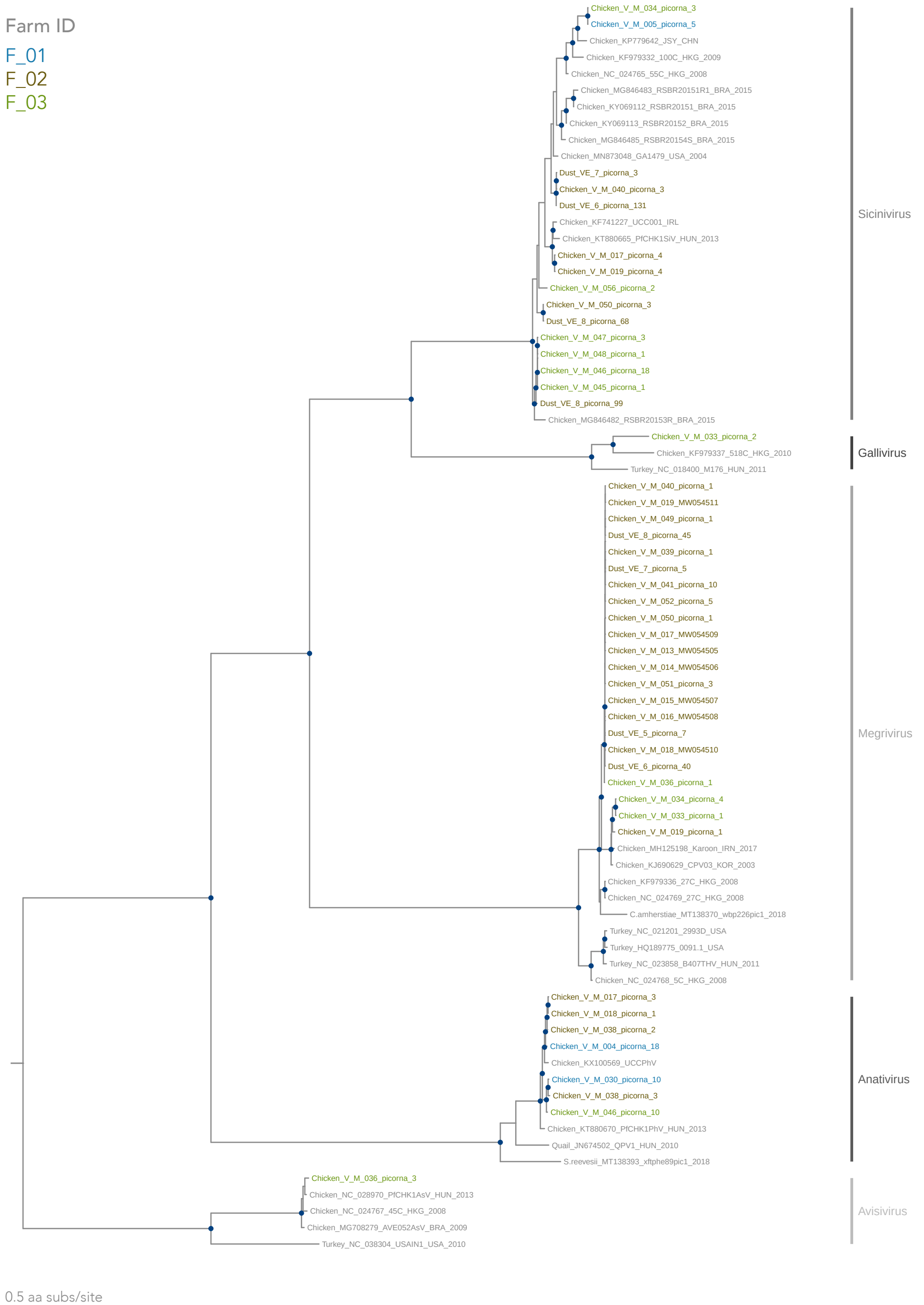
Figure S2. An overview of picornavirus genera prevalence in chicken feces and farm dust samples. Associated metadata is shown at the top panel. The color intensity of the heatmap (bottom panel) is determined by number of contigs with minimum length of 300 nt, at least 70% identity and an e-value threshold of $1x10^{-10}$ when comparing with closest reference in our database. Sample order is assorted by farm ID, sampling time point. Time point is an arbitrary number. Samples were collected at the same time and place when both Farm ID and Time point match.

Table S1. A list of corresponding BioProject, BioSample and SRA accession numbers for all samples.

Table S2. A list of corresponding GenBank accession numbers for sequences used in phylogenetic analyses.


F_01
F_02
F_03
Farm ID
Production cycle
Time point
Sample type
Astroviridae
Caliciviridae
Coronaviridae
Parvoviridae
Picobirnaviridae
Picornaviridae
Reoviridae
V_M_001
V_M_002
V_M_003
V_M_004
VE_1
V_M_005
V_M_006
V_M_007
V_M_008
VE_2
V_M_009
V_M_010
V_M_011
V_M_012
VE_3
V_M_021
V_M_022
V_M_023
V_M_024
VE_4
V_M_029
V_M_030
V_M_031
V_M_032
V_M_013
V_M_014
V_M_015
V_M_016
VE_5
V_M_017
V_M_018
V_M_019
V_M_020
VE_6
V_M_038
V_M_039
V_M_040
VE_7
V_M_041
VE_8
V_M_049
V_M_050
V_M_051
V_M_052
VE_9
V_M_025
V_M_026
V_M_027
V_M_028
VE_10
V_M_033
V_M_034
V_M_036
VE_11
V_M_045
V_M_046
V_M_047
V_M_048
VE_12
V_M_053
V_M_054
V_M_055
V_M_056
VE_13
Farm ID
F_01
F_02
F_03
Production cycle
Week 2
Week 3
Week 4
Week 5
Time point
1
2
3
4
5
Sample type
Dust
Feces
No. of contigs
(id% >= 70, length >= 300)
40
30
20
10
0


F_01
F_02
F_03
Farm ID
Production cycle
Time point
Sample type
Anativirus
Avisivirus
Gallivirus
Megrivirus
Orivirus
Sicinivirus
V_M_001
V_M_002
V_M_003
V_M_004
VE_1
V_M_005
V_M_006
V_M_007
V_M_008
VE_2
V_M_009
V_M_010
V_M_011
V_M_012
VE_3
V_M_021
V_M_022
V_M_023
V_M_024
VE_4
V_M_029
V_M_030
V_M_031
V_M_032
V_M_013
V_M_014
V_M_015
V_M_016
VE_5
V_M_017
V_M_018
V_M_019
V_M_020
VE_6
V_M_038
V_M_039
V_M_040
VE_7
V_M_041
VE_8
V_M_049
V_M_050
V_M_051
V_M_052
VE_9
V_M_025
V_M_026
V_M_027
V_M_028
VE_10
V_M_033
V_M_034
V_M_036
VE_11
V_M_045
V_M_046
V_M_047
V_M_048
VE_12
V_M_053
V_M_054
V_M_055
V_M_056
VE_13
Farm ID
F_01
F_02
F_03
Production cycle
Week 2
Week 3
Week 4
Week 5
Time point
1
2
3
4
5
Sample type
Dust
Feces
No. of contigs
(id% >= 70, length >= 300)
40
30
20
10
0


Farm ID
F_01
F_02
F_03
Chicken_V_M_034_picorna_3
Chicken_V_M_005_picorna_5
Chicken_KP779642_JSY_CHN
Chicken_KF979332_100C_HKG_2009
Chicken_NC_024765_55C_HKG_2008
Chicken_MG846483_RSBR20151R1_BRA_2015
Chicken_KY069112_RSBR20151_BRA_2015
Chicken_KY069113_RSBR20152_BRA_2015
Chicken_MG846485_RSBR20154S_BRA_2015
Chicken_MN873048_GA1479_USA_2004
Dust_VE_7_picorna_3
Chicken_V_M_040_picorna_3
Dust_VE_6_picorna_131
Chicken_KF741227_UCC001_IRL
Chicken_KT880665_PfCHK1SiV_HUN_2013
Chicken_V_M_017_picorna_4
Chicken_V_M_019_picorna_4
Chicken_V_M_056_picorna_2
Chicken_V_M_050_picorna_3
Dust_VE_8_picorna_68
Chicken_V_M_047_picorna_3
Chicken_V_M_048_picorna_1
Chicken_V_M_046_picorna_18
Chicken_V_M_045_picorna_1
Dust_VE_8_picorna_99
Chicken_MG846482_RSBR20153R_BRA_2015
Sicinivirus
Chicken_V_M_033_picorna_2
Chicken_KF979337_518C_HKG_2010
Turkey_NC_018400_M176_HUN_2011
Gallivirus
Chicken_V_M_040_picorna_1
Chicken_V_M_019_MW054511
Chicken_V_M_049_picorna_1
Dust_VE_8_picorna_45
Chicken_V_M_039_picorna_1
Dust_VE_7_picorna_5
Chicken_V_M_041_picorna_10
Chicken_V_M_052_picorna_5
Chicken_V_M_050_picorna_1
Chicken_V_M_017_MW054509
Chicken_V_M_013_MW054505
Chicken_V_M_014_MW054506
Chicken_V_M_051_picorna_3
Chicken_V_M_015_MW054507
Chicken_V_M_016_MW054508
Dust_VE_5_picorna_7
Chicken_V_M_018_MW054510
Dust_VE_6_picorna_40
Chicken_V_M_036_picorna_1
Chicken_V_M_034_picorna_4
Chicken_V_M_033_picorna_1
Chicken_V_M_019_picorna_1
Chicken_MH125198_Karoon_IRN_2017
Chicken_KJ690629_CPV03_KOR_2003
Chicken_KF979336_27C_HKG_2008
Chicken_NC_024769_27C_HKG_2008
C.amherstiae_MT138370_wbp226pic1_2018
Turkey_NC_021201_2993D_USA
Turkey_HQ189775_0091.1_USA
Turkey_NC_023858_B407THV_HUN_2011
Chicken_NC_024768_5C_HKG_2008
Megrivirus
Chicken_V_M_017_picorna_3
Chicken_V_M_018_picorna_1
Chicken_V_M_038_picorna_2
Chicken_V_M_004_picorna_18
Chicken_KX100569_UCCPhV
Chicken_V_M_030_picorna_10
Chicken_V_M_038_picorna_3
Chicken_V_M_046_picorna_10
Chicken_KT880670_PfCHK1PhV_HUN_2013
Quail_JN674502_QPV1_HUN_2010
S.reevesii_MT138393_xftphe89pic1_2018
Anativirus
Chicken_V_M_036_picorna_3
Chicken_NC_028970_PfCHK1AsV_HUN_2013
Chicken_NC_024767_45C_HKG_2008
Chicken_MG708279_AVE052AsV_BRA_2009
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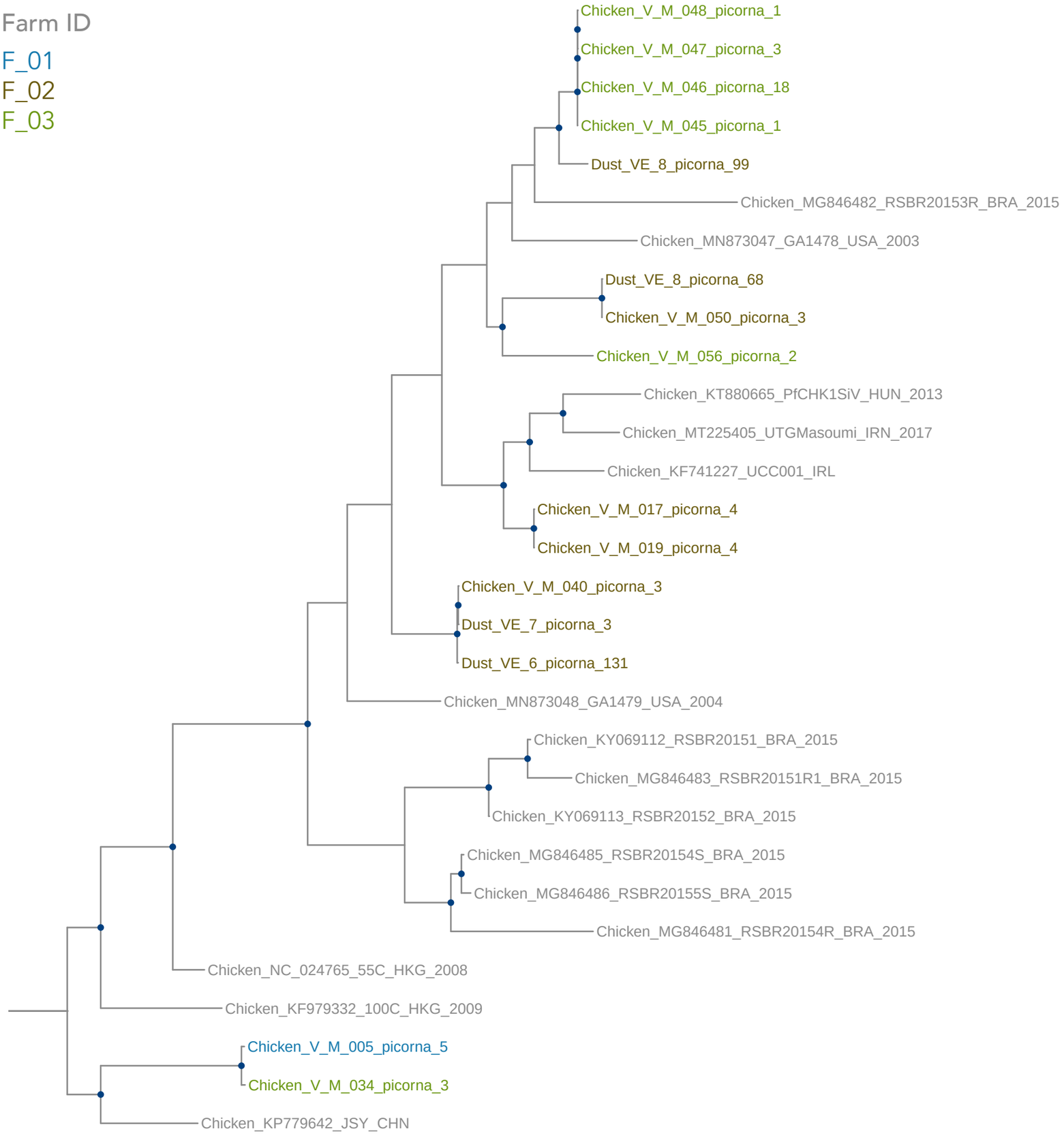
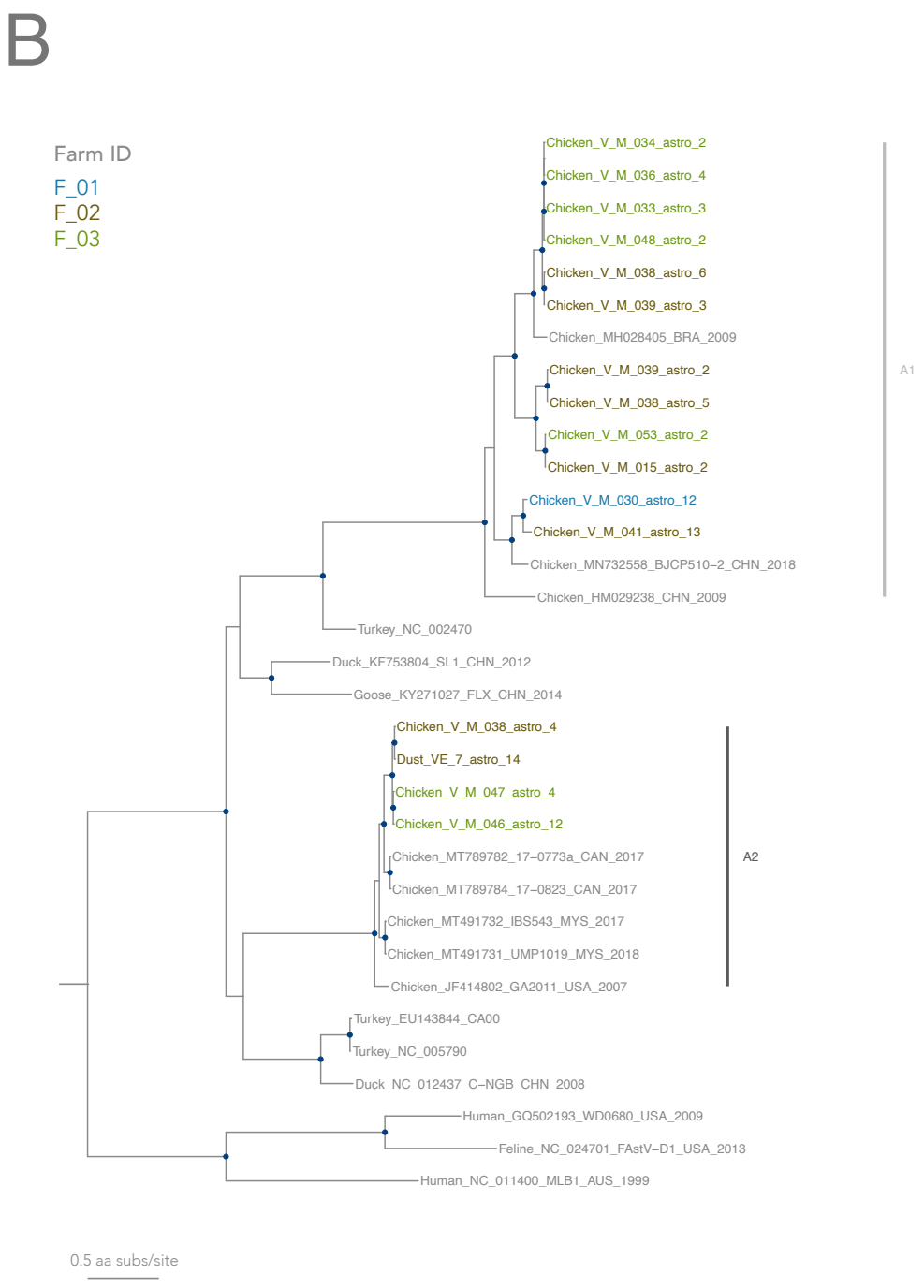
Avisivirus
0.5 aa subs/site


Farm ID
F_01
F_02
F_03
Chicken_V_M_048_picorna_1
Chicken_V_M_047_picorna_3
Chicken_V_M_046_picorna_18
Chicken_V_M_045_picorna_1
Dust_VE_8_picorna_99
Chicken_MG846482_RSBR20153R_BRA_2015
Chicken_MN873047_GA1478_USA_2003
Dust_VE_8_picorna_68
Chicken_V_M_050_picorna_3
Chicken_V_M_056_picorna_2
Chicken_KT880665_PfCHK1SiV_HUN_2013
Chicken_MT225405_UTGMasoumi_IRN_2017
Chicken_KF741227_UCC001_IRL
Chicken_V_M_017_picorna_4
Chicken_V_M_019_picorna_4
Chicken_V_M_040_picorna_3
Dust_VE_7_picorna_3
Dust_VE_6_picorna_131
Chicken_MN873048_GA1479_USA_2004
Chicken_KY069112_RSBR20151_BRA_2015
Chicken_MG846483_RSBR20151R1_BRA_2015
Chicken_KY069113_RSBR20152_BRA_2015
Chicken_MG846485_RSBR20154S_BRA_2015
Chicken_MG846486_RSBR20155S_BRA_2015
Chicken_MG846481_RSBR20154R_BRA_2015
Chicken_NC_024765_55C_HKG_2008
Chicken_KF979332_100C_HKG_2009
Chicken_V_M_005_picorna_5
Chicken_V_M_034_picorna_3
Chicken_KP779642_JSY_CHN
0.2 nt subs/site

A
Farm ID
F_01
F_02
F_03
Chicken_V_M_036_astro_4
Chicken_V_M_048_astro_2
Chicken_V_M_034_astro_2
Chicken_V_M_033_astro_3
Chicken_HM029238_CHN_2009
Chicken_MN732558_BJCP510–2_CHN_2018
Chicken_MH028405_BRA_2009
Chicken_V_M_038_astro_5
Chicken_V_M_039_astro_2
Chicken_V_M_030_astro_12
Chicken_V_M_038_astro_6
Chicken_V_M_039_astro_3
Chicken_V_M_053_astro_2
Chicken_V_M_015_astro_2
Chicken_V_M_041_astro_13
A1
Chicken_V_M_046_astro_12
Chicken_V_M_047_astro_4
Chicken_V_M_038_astro_4
Dust_VE_7_astro_14
Chicken_MT491731_UMP1019_MYS_2018
Chicken_MT491732_IBS543_MYS_2017
Chicken_MT789784_17–0823_CAN_2017
Chicken_MT789782_17–0773a_CAN_2017
Chicken_JF414802_GA2011_USA_2007
A2
Turkey_NC_005790
Turkey_EU143844_CA00
Duck_KF753804_SL1_CHN_2012
Duck_NC_012437_C–NGB_CHN_2008
Goose_KY271027_FLX_CHN_2014
Turkey_NC_002470
Human_GQ502193_WD0680_USA_2009
Feline_NC_024701_FAstV–D1_USA_2013
Human_NC_011400_MLB1_AUS_1999
0.5 aa subs/site


B
Farm ID
F_01
F_02
F_03
Chicken_V_M_034_astro_2
Chicken_V_M_036_astro_4
Chicken_V_M_033_astro_3
Chicken_V_M_048_astro_2
Chicken_V_M_038_astro_6
Chicken_V_M_039_astro_3
Chicken_MH028405_BRA_2009
Chicken_V_M_039_astro_2
Chicken_V_M_038_astro_5
Chicken_V_M_053_astro_2
Chicken_V_M_015_astro_2
Chicken_V_M_030_astro_12
Chicken_V_M_041_astro_13
Chicken_MN732558_BJCP510–2_CHN_2018
Chicken_HM029238_CHN_2009
A1
Turkey_NC_002470
Duck_KF753804_SL1_CHN_2012
Goose_KY271027_FLX_CHN_2014
Chicken_V_M_038_astro_4
Dust_VE_7_astro_14
Chicken_V_M_047_astro_4
Chicken_V_M_046_astro_12
Chicken_MT789782_17–0773a_CAN_2017
Chicken_MT789784_17–0823_CAN_2017
Chicken_MT491732_IBS543_MYS_2017
Chicken_MT491731_UMP1019_MYS_2018
Chicken_JF414802_GA2011_USA_2007
A2
Turkey_EU143844_CA00
Turkey_NC_005790
Duck_NC_012437_C–NGB_CHN_2008
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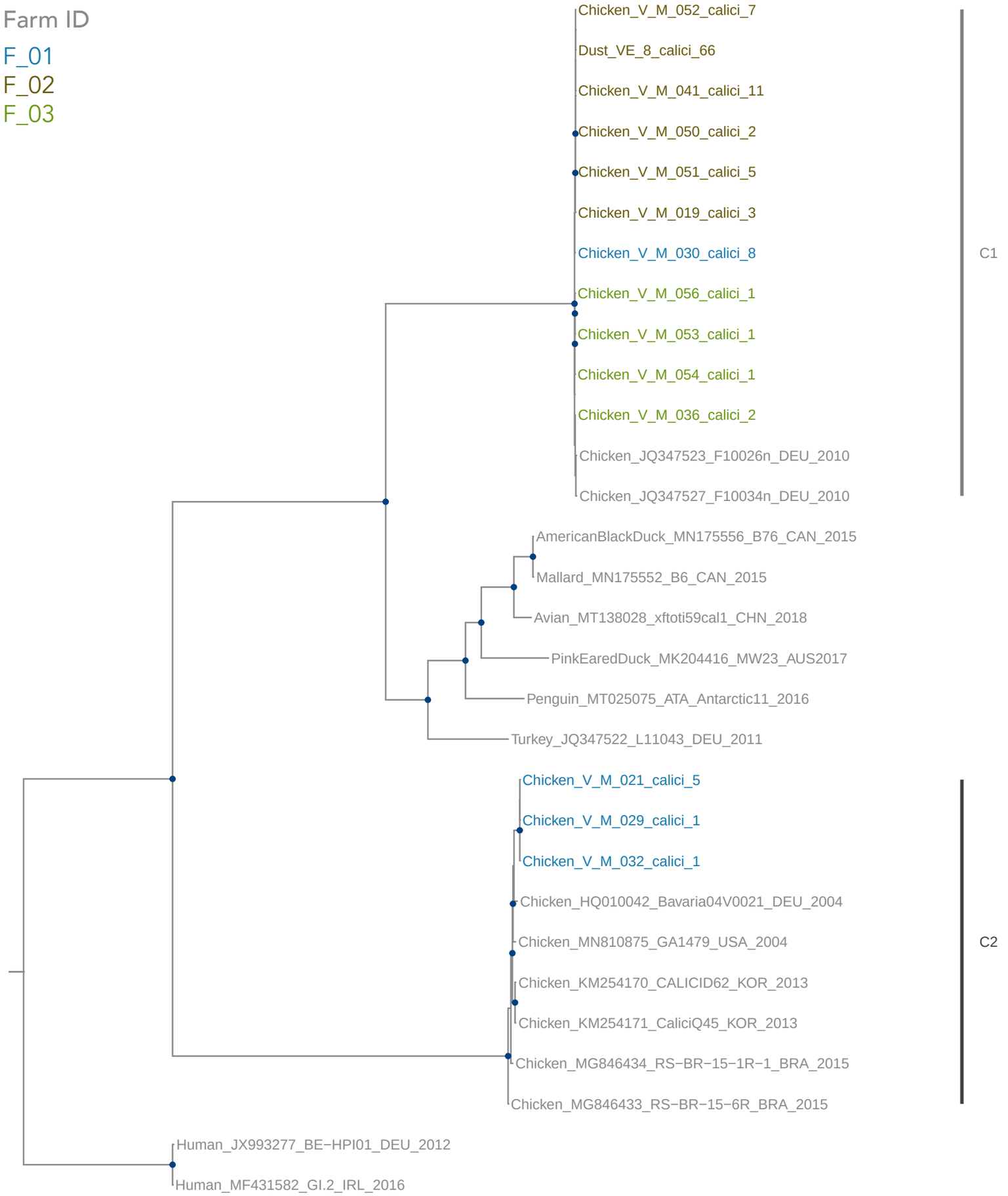
Feline_NC_024701_FAstV–D1_USA_2013
Human_NC_011400_MLB1_AUS_1999
0.5 aa subs/site


Farm ID
F_01
F_02
F_03
Chicken_V_M_052_calici_7
Dust_VE_8_calici_66
Chicken_V_M_041_calici_11
Chicken_V_M_050_calici_2
Chicken_V_M_051_calici_5
Chicken_V_M_019_calici_3
Chicken_V_M_030_calici_8
Chicken_V_M_056_calici_1
Chicken_V_M_053_calici_1
Chicken_V_M_054_calici_1
Chicken_V_M_036_calici_2
Chicken_JQ347523_F10026n_DEU_2010
Chicken_JQ347527_F10034n_DEU_2010
C1
AmericanBlackDuck_MN175556_B76_CAN_2015
Mallard_MN175552_B6_CAN_2015
Avian_MT138028_xftoti59cal1_CHN_2018
PinkEaredDuck_MK204416_MW23_AUS2017
Penguin_MT025075_ATA_Antarctic11_2016
Turkey_JQ347522_L11043_DEU_2011
Chicken_V_M_021_calici_5
Chicken_V_M_029_calici_1
Chicken_V_M_032_calici_1
Chicken_HQ010042_Bavaria04V0021_DEU_2004
Chicken_MN810875_GA1479_USA_2004
Chicken_KM254170_CALICID62_KOR_2013
Chicken_KM254171_CaliciQ45_KOR_2013
Chicken_MG846434_RS−BR−15−1R−1_BRA_2015
Chicken_MG846433_RS−BR−15−6R_BRA_2015
C2
Human_JX993277_BE−HPI01_DEU_2012
Human_MF431582_GI.2_IRL_2016
0.5 aa subs/site