

1 **Divergent influenza-like viruses of amphibians and fish support** 2 **an ancient evolutionary association**

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23 **Abstract**

24 Influenza viruses (family *Orthomyxoviridae*) infect a variety of vertebrates, including birds, humans,
25 and other mammals. Recent metatranscriptomic studies have uncovered divergent influenza viruses in
26 amphibians, fish and jawless vertebrates, suggesting that these viruses may be widely distributed. We
27 sought to identify additional vertebrate influenza-like viruses through the analysis of publicly
28 available RNA sequencing data. Accordingly, by data mining, we identified the complete coding
29 segments of five divergent vertebrate influenza-like viruses. Three fell as sister lineages to influenza B
30 virus: salamander influenza-like virus in Mexican walking fish (*Ambystoma mexicanum*) and plateau
31 tiger salamander (*Ambystoma velasci*), siamese algae-eater influenza-like virus in siamese algae-eater
32 fish (*Gyrinocheilus aymonieri*) and chum salmon influenza-like virus in chum salmon (*Oncorhynchus*
33 *keta*). Similarly, we identified two influenza-like viruses of amphibians that fell as sister lineages to
34 influenza D virus: cane toad influenza-like virus and the ornate chorus frog influenza-like virus, in the
35 cane toad (*Rhinella marina*) and ornate chorus frog (*Microhyla fissipes*), respectively. Despite their
36 divergent phylogenetic positions, these viruses retained segment conservation and splicing consistent
37 with transcriptional regulation in influenza B and influenza D viruses, and were detected in respiratory
38 tissues. These data suggest that influenza viruses have been associated with vertebrates for their entire
39 evolutionary history.

40 **Keywords:** *Orthomyxoviridae*; influenza; metatranscriptomics; fish; amphibians; evolution;
41 phylogeny

42

43 1. Introduction

44 Influenza viruses are multi-segmented, negative-sense RNA viruses of the family
45 *Orthomyxoviridae*, which also includes the genera *Quaranjavirus*, *Isavirus* and *Thogotovirus*, as well
46 as a wide diversity of orthomyxo-like viruses found in invertebrates [1-3]. Until recently, influenza
47 viruses had been limited to a small group of pathogens with public health importance for humans,
48 particularly those of pandemic and epidemic potential – influenza A virus (IAV, *Alphainfluenzavirus*)
49 and influenza B virus (IBV, *Betainfluenzavirus*). Influenza A virus is an important, multi-host virus
50 which is best described in humans, pigs, horses and birds [4-7]. These viruses are epidemic in humans
51 and are closely monitored for pandemic potential. Influenza B viruses similarly cause yearly epidemics
52 in humans [8], and their detection in both seals [9] and pigs [10] highlights the potential for non-human
53 IBV reservoirs.

54
55 While IAV and IBV are subject to extensive research, far less is known about two additional
56 influenza viruses: Influenza C virus (ICV, *Gammainfluenzavirus*) and Influenza D virus (IDV,
57 *Deltainfluenzavirus*). Influenza C virus has been found in humans worldwide and is associated with
58 mild clinical outcomes [11, 12]. Based on its detection in pigs, dogs and camels, it is likely that this
59 virus has a zoonotic origin [13-17]. Influenza D virus, first identified in 2015 [18], is currently thought
60 to have a limited host range and is primarily associated with cattle and small ruminants [18-20], pigs
61 [18] as well as dromedary camels [17]. Although there is no record of active infection in humans, there
62 are indications of past infection based on serological studies [12, 21, 22].

63
64 Despite intensive research on the characterisation of influenza viruses, our understanding of their
65 true diversity and their evolutionary history is undoubtedly limited. Of critical importance are recent
66 meta-transcriptomic (i.e. bulk RNA sequencing) studies that report the discovery of novel "influenza-
67 like" viruses from both amphibian and fish hosts – Wuhan spiny eel influenza virus, Wuhan asiatic toad
68 influenza virus and Wenling hagfish influenza virus [3]. Such a phylogenetic pattern, particularly the
69 position of the hagfish (jawless vertebrate) influenza virus as the sister-group to the other vertebrate
70 influenza viruses, is compatible with virus-host co-divergence over millions of years. This, combined
71 with relatively frequent cross-species transmission, suggests that a very large number of animal
72 influenza-like viruses remain to be discovered.

73 Given the previous success of data-mining to identify a range of novel viruses, including
74 rhabdoviruses [23], flaviviruses [24] and parvoviruses [25], we hypothesised we could mine publicly

75 available transcriptome databases for evidence of undescribed vertebrate influenza viruses and that this
76 would provide further insights into their evolutionary origins. Given the highly under-sampled nature
77 of "lower" vertebrate hosts, such as fish and amphibians, we focused on data from these taxa. From
78 this, we identified five novel influenza-like viruses that provide new insights into the evolution and
79 host range of this important group of animal viruses.

80 **2. Materials and Methods**

81 *2.1 Identification of divergent influenza-like viruses from vertebrate de novo transcriptome assemblies*

82 To identify novel and potentially divergent vertebrate influenza viruses we screened *de novo*
83 transcriptome assemblies available at the National Center for Biotechnology Information (NCBI)
84 Transcriptome Shotgun Assembly (TSA) Database (<https://www.ncbi.nlm.nih.gov/genbank/tsa/>) and
85 the China National GeneBank (CNGB) Fish-T1K (Transcriptomes of 1,000 Fishes) Project database
86 (<https://db.cngb.org>) [26]. Amino acid sequences of the influenza virus reference strains - IAV
87 A/Puerto Rico/8/34 (H1N1), IBV (B/Lee/1940), ICV (C/Ann Arbor/1/50) and IDV
88 (D/bovine/France/2986/2012) - were queried against the assemblies using the translated Basic Local
89 Alignment Search Tool (tBLASTn) algorithm under default scoring parameters and the BLOSUM45
90 matrix. For the TSA, we restricted the search to the Vertebrata (taxonomic identifier [taxid] 7742).
91 Putative influenza-like virus contigs were subsequently queried using BLASTx against the non-
92 redundant virus database. Two fish transcriptomes (BioProjects PRJNA329073 and PRJNA359138)
93 were excluded because of the presence of reads that were near identical to IAV, strongly suggestive of
94 contamination.

95 *2.2 Recovery of additional influenza-like virus segments through de novo assembly and coverage* 96 *statistics*

97 Putative influenza-like viruses identified in the transcriptome data were queried against genus-
98 wide RNA-Seq samples deposited in the Sequence Read Archive (SRA) using the BLASTn tool. Raw
99 fastq files originating from transcriptome sequencing libraries were downloaded and imported to the
100 Galaxy Australia web server (<https://usegalaxy.org.au/>, v. 19.05). Sequencing adapters were identified
101 using FastQC, and reads were quality trimmed using Trimmomatic (Galaxy v. 0.36.4) under the
102 following conditions: sliding window = 4 and average quality = 20 [27]. For the recovery of full-length
103 transcripts corresponding to putative influenza-like viruses, clean reads were then *de novo* assembled
104 using Trinity (Galaxy Version 2.9.1) [28]. For virus genome statistics, clean reads were re-mapped

105 against virus segments using the Burrow-Wheeler Aligner (BWA-MEM Galaxy Version 0.7.17.1)
106 under default conditions, and the resultant binary alignment file was analysed with the bedtools
107 (v2.27.1) genome coverage tool [29]. For the production of the salamander influenza-virus infection
108 heat map of *Ambystoma velasci* tissues, quantitation of abundance for each library was calculated using
109 the proportion of total mapped salamander influenza-like reads for each library and heatmaps were
110 produced using R v3.5.3 integrated in RStudio v1.1.463 and *ggplot2*.

111 *2.3 Influenza virus genome annotation*

112 Viral open reading frames were predicted using ORFFinder
113 (<https://www.ncbi.nlm.nih.gov/orffinder/>). To characterise functional domains, predicted protein
114 sequences were subjected to a domain-based search using the Conserved Domain Database version
115 3.16 (<https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>) and cross-referenced with the PFam
116 database (version 32.0) hosted at (<http://pfam.xfam.org/>). Transmembrane topology prediction using
117 the TMHMM Server version 2.0 (www.cbs.dtu.dk/services/TMHMM/). For the identification of
118 potential splice sites in influenza-like viruses we used alternative isoforms assembled using Trinity and
119 then manually validated for donor and acceptor sites using the Alternative Splice Site Predictor (ASSP)
120 (<http://wangcomputing.com/assp/index.html>) [30] for NS and M segments guided by experimentally
121 validated positions in IAV/IBV/ICV (Reviewed here [31]). Viral genome sequences have been
122 deposited in GenBank under the accession numbers: MT926372-MT926409

123 *2.4 Phylogenetic analysis*

124 Multiple sequence alignments of predicted influenza virus protein sequences were performed using
125 MAFFT-L-INS-i (version 7.471) [32]. Ambiguously aligned regions were removed using TrimAl (v.
126 1.3) under the automated 1 method [33]. Individual protein alignments were then analysed to determine
127 the best-fit model of amino acid substitution according to the Bayesian Information Criterion using
128 ModelFinder [34] incorporated in IQ-TREE (Version 2.1.1) [35] and excluding the invariant sites
129 parameter. For the PB1, PA, NP segments the Le-Gascuel (LG) model [36] with discrete gamma model
130 with 4 rate categories (+ Γ_4) and empirical amino acid frequencies (+F) was selected as the most
131 suitable. For the HA/HEF alignments, the Whelan and Goldman (WAG) model [37] with a discrete
132 gamma model with 4 rate categories (+ G_4) was selected. For the NA alignment, the FLU+ G_4 model
133 was identified [38], and finally, PB2 and NS (LG+ G_4). For the M1/M2 alignment, LG + FreeRate
134 model (+R3) [39] was selected. Maximum likelihood trees were then inferred using IQ-TREE (Version

135 2.1.1) with Ultrafast bootstrap approximation (n=40,000) [40]. Resultant consensus tree from combined
136 bootstrap trees were visualised using FigTree version 1.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

137 3. Results

138 3.1 *Discovery and annotation of novel influenza-like viruses in vertebrates*

139 We initially screened 776 vertebrate *de novo* assembled transcriptomes deposited in the
140 Transcriptome Shotgun Assembly Sequence Database (TSA) and a further 158 transcriptomes
141 available on the Fish-T1K Project. Within the TSA entries, the most abundant assemblies were from
142 the bony fish (*Actinopterygii*), accounting for 346 transcriptomes. We also screened the amphibian
143 (n=57) and cartilaginous fish (n=9) transcriptomes. From these libraries, we detected influenza-like
144 viruses in 0.6% of the bony fish libraries (n=2) and in 7% (n=4) of the amphibian libraries.

145
146 Our transcriptome mining identified fragments of divergent IBV-like Polymerase Basic 1 and 2
147 (PB1, PB2) segments from an unpublished transcriptome of ear and neuromast samples from an
148 *Ambystoma mexicanum* (Mexican walking fish or Axolotl) laboratory colony housed by The University
149 of Iowa (BioProject Accession: PRJNA480225). BLASTx analysis of these IBV-like fragments
150 revealed 76.32% amino acid identity to the PB1 gene of IBV (Top hit Genbank accession
151 ANW79211.1; E-value: 0; query cover 100%) and 60.94% to the IBV PB2 (Top hit Genbank
152 accession: AGX15674.1; E-value: 2e-49; query cover: 83%) (Table 1). To recover additional segments
153 and full-length PB1 and PB2 genes, we used BLASTn to screen 2,037 *Ambystoma* RNA-Seq libraries
154 from several *Ambystoma* species available on the SRA. Accordingly, we were able to identify this
155 tentatively named salamander influenza-like virus in 139/2037 (6.8%) of screened libraries. BLASTn
156 positive libraries were subsequently *de novo* assembled, allowing the recovery of all eight segments of
157 this virus (Figure 1; Table 1). In addition to *A. mexicanum* samples infected with salamander influenza-
158 like virus we identified a smaller number of reads from Anderson's salamander (*Ambystoma*
159 *andersoni*) and spotted salamander (*Ambystoma maculatum*) libraries [42]. Finally, we identified a
160 variant of salamander influenza virus from RNA-Seq data generated from laboratory colonies of
161 another neotenic salamander, plateau tiger salamander (*Ambystoma velasci*) [41] with nucleotide
162 sequence identity between both variants of all segments between 89-94% at the nucleotide level (PB1
163 90%, PB2 91%, PA 90%, NP 91%, NA 90%, HA 89%, NS 94%, M 93%) (Table 1). The presence of
164 infection in up to four species of *Ambystoma* colonies indicates the potential for a wide host range for
165 this virus across this host genus. Additionally, as the majority of *A. mexicanum* colonies originate from

166 the *Ambystoma* Genetic Stock Center (AGSC, University of Kentucky, KY) [43-46] and in some cases
167 co-housed together with other species positive for this virus [42], this suggests that this virus may
168 circulate between numerous laboratory-reared salamander colonies.

169

170 We similarly identified an influenza virus in the Siamese algae-eater (*Gyrinocheilus aymonieri*)
171 (SRA accession: SRR5997773), tentatively termed siamese algae-eater influenza-like virus, sampled as
172 part of the Fish-T1K Project (Table 1) [26]. While there is limited meta-data available, the library was
173 prepared from gill tissues. There was no evidence of this virus in any other siamese algae-eater samples
174 available on the SRA (n=2) through BLASTn analysis.

175

176 Finally, we identified another IBV-like virus in chum salmon (*Oncorhynchus keta*), provisionally
177 termed chum salmon influenza-like virus (Table 1). While the geographic origin of the samples is
178 unknown the meta-data from this library indicates the RNA-Seq originates from gill tissues of alevin
179 stage chum salmon, transferred to the Korea Fisheries Resources Agency (FIRA) laboratory and reared
180 in tanks with recirculating freshwater [47]. We identified chum salmon influenza-like virus sequences
181 in one of the three libraries from this project (SRA Accession: SRR6998471). However, all segments
182 were convincing *de novo* assembled and covered through re-mapping with reads corresponding to
183 0.045% of the library (45,151/100,244,990 mapped reads).

184

185 In addition to novel viruses related to IBV, we identified two influenza viruses of amphibians that
186 exhibited high pairwise amino acid identity to segments from IDV and ICV (Table 1). This result is
187 particularly noteworthy as the previously identified amphibian influenza virus, wuhan asiatic toad
188 influenza virus, was more closely related to IBV [3]. Cane toad influenza-like virus was identified in
189 four larval samples of cane toad (*Rhinella marina*), from two geographic locations in Australia,
190 Innisfail, Queensland, Australia (SRA accessions: SRR5446725, SRR5446726) and Oombulgurri,
191 Western Australia, Australia (SRA accessions: SRR5446727, SRR5446728) [48]. We did not identify
192 any virus reads in any adult tissue samples taken in Australia nor from libraries constructed from cane
193 toad samples from Macapa city, Brazil, from the same study [48]. Similarly, we did not recover any
194 cane toad influenza-like virus reads in any of the 16 liver tissue samples from Australia reported in
195 another metatranscriptomic study, which aimed to reveal the virome of the cane toad [49].

196

197 The final IDV-related virus identified was tentatively named ornate chorus frog influenza-like
198 virus detected in ornate chorus frog (*Microhyla fissipes*) (Table 1). This virus was identified in two

199 sequencing projects, both of which were from samples of ornate chorus frog collected from paddy
200 fields in Chengdu, China, albeit at different times; May 2014 (Bioproject accession: PRJNA295354)
201 [50] and June 2016 (Bioproject accession: PRJNA386601)[51]. Between both data sets, the largest
202 number of ornate chorus frog influenza-like virus reads originated from the lung tissue of the stage 28
203 tadpole (SRA accession: SRR5557878) in the second study (June 2016 [51]), representing 0.13% of all
204 reads (81,860/ 61,952,548). While there were fewer reads mapping to the ornate chorus frog influenza-
205 like virus in whole tissues of three developmental stages in the study carried out in 2014 [50]. In the
206 whole organism samples the highest viral abundance was in the metamorphic climax (1,462/59,993,514
207 reads) (SRA accession: SRR2418623), and pre-metamorphosis developmental phases
208 (1,113/63,073,925 reads) (SRA accession: SRR2418554) with only 7 reads were identified in the
209 complete metamorphosis library (7/56,000,000 reads) (SRA accession: SRR2418812). The nucleotide
210 identity of ornate chorus frog influenza-like virus variants between the 2014 [50] and June 2016 [51]
211 data sets was between 93.36%-97.10% for all segments (PB1 96.98% PB2 93.95% P3 93.36% NP
212 93.83% HEF 91.87% NS 97.10% M 96.29%).

213 3.2 *The genome organisation and transcription of novel influenza-like virus genes are highly* 214 *conserved*

215 Through a combination of *de novo* assembly and tBLASTn analysis we were able to identify and
216 assemble the complete coding segments of all novel influenza-like genomes described in this study
217 (Figure 1, Figure S1-S5, Table S1-S6). For the salamander influenza-like virus, siamese algae eater
218 influenza-like virus and chum salmon influenza-like viruses, all eight segments with genome
219 arrangements similar to that of IAV and IBV were identified (Figure 1). Prediction of the ORFs from
220 all segments and protein domain analyses suggested homology between these putative virus proteins
221 (Table S1-S4, Figure S1-S3). Additionally, we identified the glycoprotein NB, which is encoded
222 through a polycistronic mRNA of the NA segment of IBVs (Figure 1). The M2 domain of chum
223 salmon influenza-like was not identified through BLASTp, likely due to high levels of sequence
224 divergence. However, we did identify the N terminus using a domain search (Table S3). In contrast, we
225 identified seven segments for each of cane toad influenza-like virus and ornate chorus frog influenza-
226 like viruses, which are characteristic of ICV/IDV encoding 7 segments (Figure 1). We recovered all
227 ORFs expected of viruses similar to ICV and IDV (Table S5-S6, Figure S4-S5).

228

229 There were a number of important differences in the composition of segments among IAV/IBV
230 and ICV/IDV. For example, segment 3 is differentiated into “PA” or “P3” by isoelectric points. The

231 isoelectric point of ornate chorus frog influenza-like virus (~6.3) and cane toad influenza-like virus
232 (~6) are similar to those of the P3 segment of ICV/IDV, while those of salamander influenza-like virus
233 (~5.6), Siamese algae eater influenza-like virus (~5.3) and chum salmon influenza-like viruses (~5.4)
234 had isoelectric points more consistent with PA of IAV (~5.4) and IBV (~5.5). With the exception of
235 chum salmon influenza-like virus, we were able to assemble two discrete isoforms of the NS gene in all
236 our assemblies and manually validate the splice junctions as *bona fide* through the identification of
237 splicing donor and acceptor sites (Table S2-6). The M segment in ICV/IDV related viruses is also post-
238 transcriptionally spliced to encode the M1 protein, and we were able to assemble two isoforms of the M
239 segment from cane toad influenza-like virus and ornate chorus frog influenza-like virus.

240 3.3 *Phylogenetic analysis of novel influenza-like viruses suggests a history of genomic reassortment*

241 One of the most striking observations of our study was that three novel influenza viruses from
242 amphibians and fish fell basal to IBV in the phylogenetic analysis, although none of the relevant
243 bootstrap values were exceptionally high (i.e. <75%) (Figure 2), and with pairwise amino acid
244 sequence similarities to the PB1 of IBV ranging from 67- 75%. The placement of these viruses, in
245 addition to Wuhan spiny eel influenza virus, at the base of the IBV lineage, strongly suggests that these
246 viruses have a long evolutionary history in vertebrates. Indeed, it is notable that salamander influenza-
247 like virus is the now the closest animal relative to IBV. Similarly, two other influenza-like viruses from
248 amphibians fell basal to IDV, this time with strong (>90% bootstrap support) and exhibited 77-80%
249 amino acid similarity in the PB1 segment.

250 These novel influenza viruses largely occupy consistent phylogenetic placements across
251 segments (Figure 3). However, chum salmon influenza-like virus is the sister-group to IBV in the
252 polymerase (PB2, PB1, PA) and NA segments, but is the sister-group to both IAV/IBV in the NP
253 segment. Also of note was that the two amphibian influenza-like genomes are the sister lineages to IDV
254 in all segments with the exception of the HEF, in which they are the sister lineages to the clade
255 containing ICV/IDV.

256 3.4 *Multi-tissue RNA-sequencing of developmental Plateau tiger salamander libraries suggests* 257 *conserved influenza virus tropism in vertebrates.*

258 To gain further insight into the tissue tropism of these divergent influenza viruses, we screened
259 libraries from a multi-tissue and developmental library of the plateau tiger salamander. This data set
260 comprised of 86 individual samples from gills, hearts and lungs at different stages of

261 premetamorphosis, prometamorphosis, metamorphosis and metamorphosis late [41]. We identified
262 RNA originating from salamander influenza-like virus abundantly in the libraries originating from gills
263 at the metamorphosis late stage with an average number of the reads in all 6 libraries corresponding to
264 approximately 0.22% of all reads (Figure 4). The second most abundant tissue and developmental stage
265 was the postmetamorphosis stage lung tissue which had, on average, 0.013% of all reads correspond to
266 salamander influenza-like virus. The only other tissue and developmental stage with all segments
267 represented were the gills from the premetamorphosis stage with an average ~0.003% of all reads
268 originating from Salamander influenza-like virus.

269

270 **4. Discussion**

271 We have identified five novel influenza viruses in fish and amphibians through mining publicly
272 available meta-transcriptomic data. Despite intensive research on influenza viruses for almost a
273 century, it is only recently that these viruses have been identified in hosts other than birds and
274 mammals. In particular, the recent identification of divergent influenza-like viruses in fish and
275 amphibians [3], as well as even more divergent orthomyxoviruses in invertebrates [2], suggests that
276 divergent members of this virus family may infect a wide range of animal hosts.

277

278 These data provide valuable insights into the evolution of influenza viruses across the vertebrates.
279 First, that the phylogeny of the IBV-like viruses in part follows the phylogeny of the hosts from which
280 they are sampled, with the Salamander influenza-like viruses falling as the sister-group to the
281 mammalian viruses and the fish viruses occupying basal positions in that group as a whole. This is
282 compatible with a process of virus-host co-divergence that likely extends hundreds of millions of years,
283 albeit with relatively frequent host-jumping. Under these circumstances, it must also be the case that
284 there are many more vertebrates carry IBV-like viruses that have yet to be investigated. Similarly, we
285 identified two amphibian viruses that fall as sister lineages to IDV, again suggestive of both long-term
286 co-divergence and highly limited sampling to date. Whether the same pattern will also be true of the
287 influenza A virus group is unclear and will only be resolved with additional sampling. Finally, it is
288 notable that Wenling hagfish virus, from a jawless vertebrate (a hagfish), occupies the basal position in
289 all of the vertebrate influenza virus, again suggesting that these viruses have been in existence for
290 hundreds of millions of years.

291

292 Despite detecting divergent influenza viruses, the stability and conservation of genome segments
293 and transcriptional regulation of viral genes through splicing is conserved. For example, salamander
294 influenza-like virus, siamese algae-eater influenza-like and chum salmon influenza-like virus, which all
295 fall basal to IBV, possess eight segments encoding all the predicted/required ORFs including NB,
296 which is a glycoprotein encoded on the NA gene of IBV only [52]. Similarly, cane toad influenza and
297 ornate chorus frog influenza had only seven segments and all ORFs consistent with ICV and IDV.
298 Overall, despite finding these viruses in non-mammalian hosts, the viral structure is highly consistent
299 with other influenza viruses strongly supporting the inclusion into these viral genera.

300 These new data also raise questions of whether influenza and influenza-like viruses have the same
301 tissue tropism among all vertebrate hosts. As with other influenza viruses, we detected all novel
302 influenza viruses in respiratory tissues (i.e. gills and lungs) of their hosts: the only exception was cane
303 toad influenza-like for which we don't have tissue-associated metadata. The most robust support for the
304 conservation of influenza-like virus infection in respiratory tissues was in the analysis of the multi-
305 tissue transcriptome of the plateau tiger salamander [41]. Before and during metamorphosis,
306 salamanders rely on gills for respiration. During metamorphosis, there is a significant reduction in gill
307 length, and in post-metamorphosis they rely on lungs for respiration [41, 53]. Based on the expectation
308 that we would find these viruses in respiratory tissues, we found salamander influenza-like virus in the
309 gills of metamorphosing salamanders and the lungs following metamorphosis. This suggests that all
310 influenza viruses primarily infect the respiratory tissues, consistent with other influenza viruses [54].
311 To date, the only exception are IAV infections which infect the surface epithelium of the
312 gastrointestinal tract in wild birds [55], and gut-associated lymphoid tissue and the squamous
313 epithelium of the palatine tonsils in bats [56].

314
315 Finally, it is interesting to speculate on whether these influenza-like viruses are host specialist or
316 generalist viruses. Influenza A-D viruses have extensive host ranges, including an array of mammalian
317 species, particularly those associated with food production (i.e. cattle and swine), and in the case of
318 IAV, wild and food production associated birds (i.e. poultry) [4-7, 18-20]. Salamander influenza-like
319 virus also appears to be a multi-host virus, being found in four salamander species raised in laboratory
320 colonies, with the greatest abundance in the Mexican walking fish and plateau tiger salamander. While
321 these species have very different life-history strategies, and their ranges do not overlap in nature [57,
322 58], both are abundant in the pet trade and used in laboratory research. Thus, breeding facilities may be
323 an important source of these viruses. Whether this multi-host trait is correlated with the use of breeding

324 facilities in salamanders, a parallel for animal production systems and influenza A-D viruses is unclear.
325 Further research is certainly warranted to determine if a broad host range is a feature of all influenza
326 viruses, including those found in “lower” vertebrates.

327

328 **5. Conclusions**

329 By mining transcriptome data we identified five novel influenza-like viruses in fish and
330 amphibians. These data strongly suggest that influenza-like viruses can infect diverse classes of
331 vertebrates and that influenza viruses have been associated with vertebrates for perhaps their entire
332 evolutionary history. While the public health and economic ramifications are undoubtedly more
333 significant for mammalian influenza viruses, only by looking at other vertebrate classes and other
334 animal lineages will we fully understand the origins and evolution of this hugely important group of
335 viruses.

336

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341

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345

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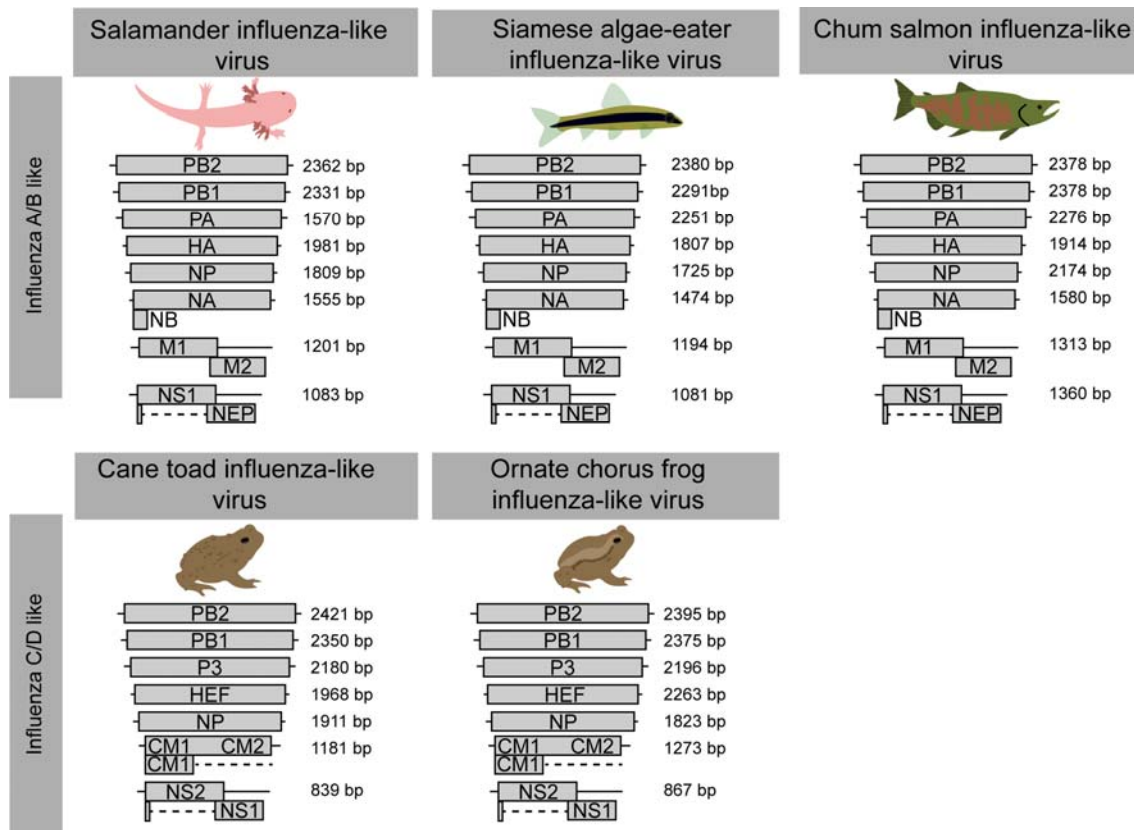
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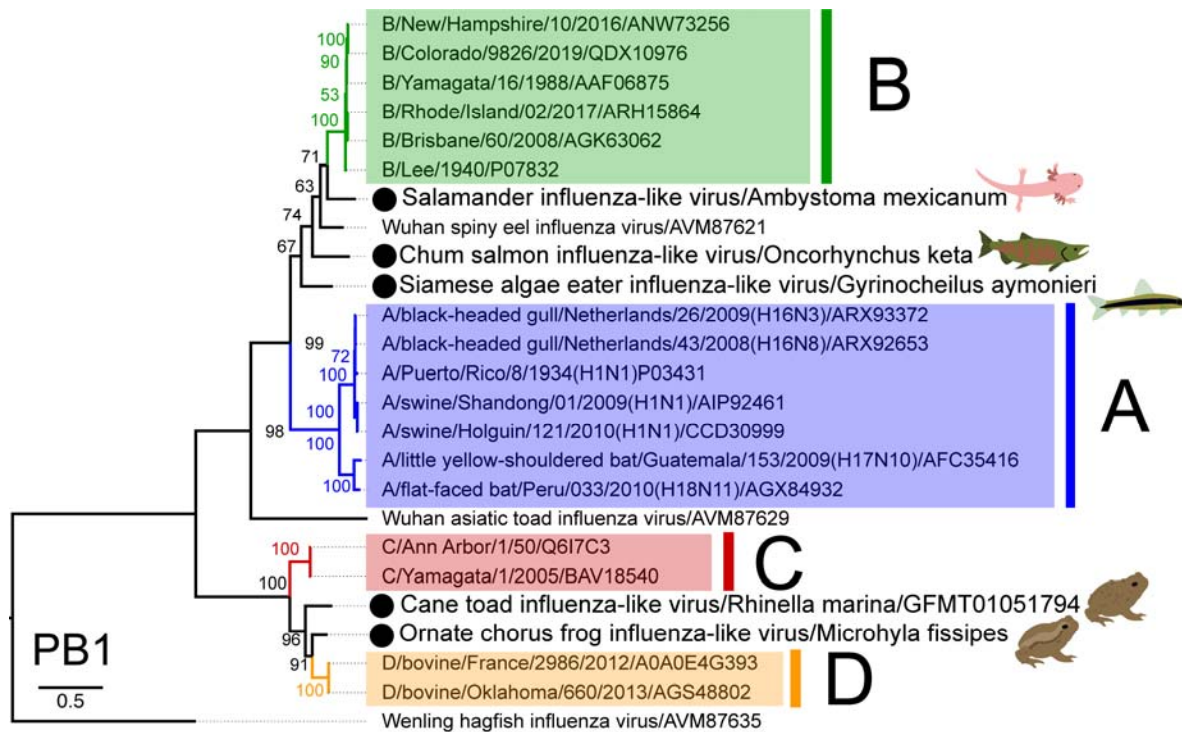
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Figure 1. Genome architecture of the novel influenza-like viruses identified here. Genome architecture of IAV/IBV and ICV/IDV is shown on the left. For each novel virus, we provide the segment name and size. PB1, RNA-dependent RNA polymerase basic subunit 1; PB2, RNA-dependent RNA polymerase basic subunit 2; PA, RNA-dependent RNA polymerase acidic subunit; NP, nucleoprotein; HA, hemagglutinin; HEF, Hemagglutinin esterase; NA, neuraminidase; M, matrix; NS, non-structural protein. Detailed annotations for each virus are presented in Table S1-S6, Figure S1-S5.

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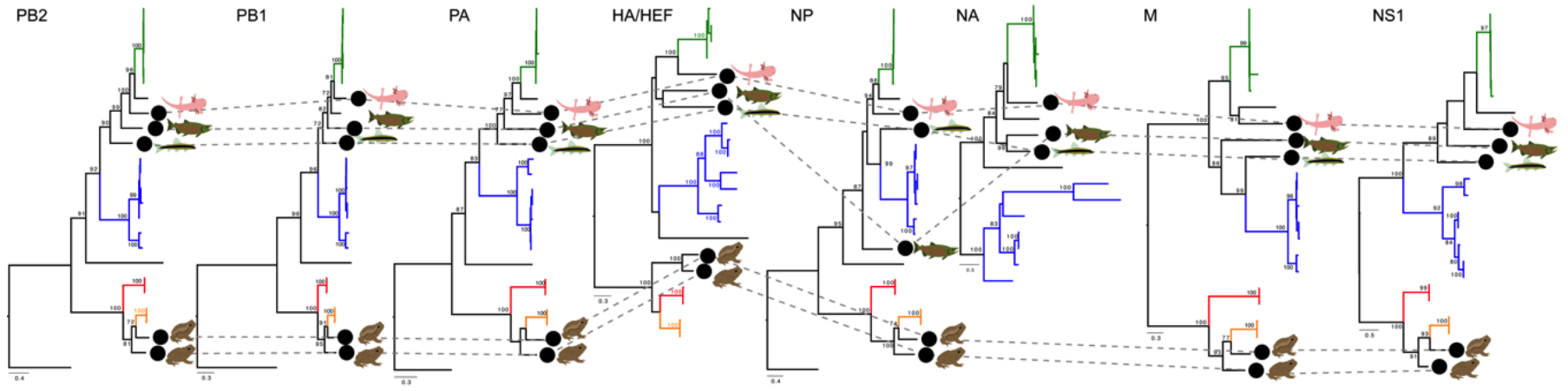
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Figure 2. The evolutionary history of vertebrate influenza-like viruses. Maximum likelihood tree of the PB1 segment, which encodes the RNA-dependent RNA polymerase, of various influenza-like viruses. Lineages corresponding to IAV, IBV, ICV, IDV are coloured blue, green, red and orange, respectively. Viruses identified in this study are denoted by a black circle and pictogram of the host species of the library. Wenling hagfish influenza virus is set as the outgroup as per Shi et al. 2018 [3]. The scale bar indicates the number of amino acid substitutions per site.



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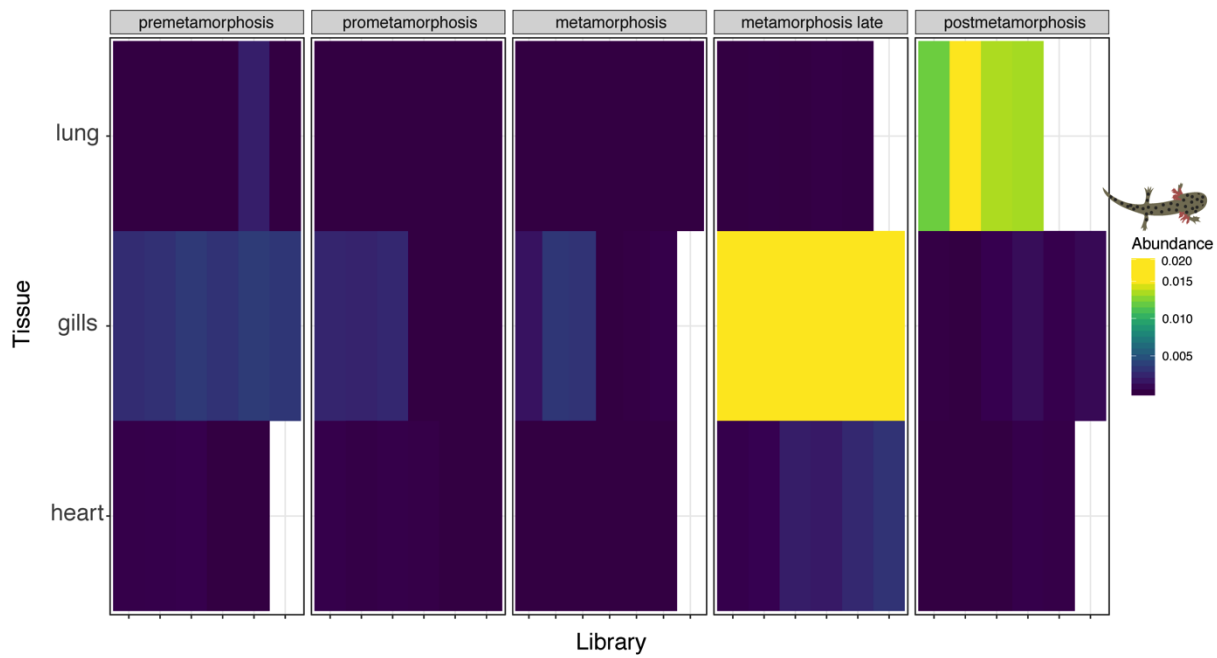
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Figure 3. Phylogenetic trees for segments in cases where complete coding genomes could be recovered. Maximum likelihood trees for each segment ordered by (segment) size. Lineages are coloured by influenza virus, in which IAV, IBV, ICV, IDV are coloured blue, green, red and orange, respectively. Viruses identified in this study are denoted by a black circle and pictogram of the host species of the library. The phylogenetic position of each virus is traced across the trees with grey dashed lines. ICV, IDV, cane toad and chorus frog influenza-like viruses do not have an NA segment. The scale bar for each tree indicates the number of amino acid substitutions per site. Where possible the trees are rooted using the hagfish influenza virus (PB2, PB1, PA/P3, NP). The HA/HEF, M and NS trees were midpoint rooted.



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543 **Figure 4.** Heat map of the abundance of Salamander influenza-like virus reads in the multi-
544 tissue transcriptome library of developmental stages of Plateau tiger salamander [41]. Read
545 abundance for this virus was highest in the gills of late metamorphosis salamanders, and lungs
546 of postmetamorphosis salamander, strongly suggesting tropism for respiratory epithelium.

Table 1. Metadata for the novel influenza viruses identified in this study.

Virus name	Host	Tissue sampled	Reference	BLASTp output of predicted amino acid identity of polymerase genes			
				Segment	GenBank Accession	Coverage (%); Identity (%)	E-value
Salamander influenza-like virus	Mexican walking fish (<i>Ambystoma mexicanum</i>)	Various	[42-46]	PB1 Influenza B virus (B/California/24/2016)	QHI05420	100%; 75.70%	0.0
	Plateau tiger salamander (<i>Ambystoma velasci</i>)	Various	[41]	PB2 Influenza B virus (B/Sydney/19/2011) PA Influenza B virus (B/Indiana/07/2016)	AZY32600 ANW74127	99%; 61.83% 97%; 59.64%	0.0 0.0
Siamese algae-eater influenza-like virus	Siamese algae-eater (<i>Gyrinocheilus aymonieri</i>)	Gills	[26]	PB1 Influenza B virus (B/California/24/2016) PB2 Influenza B virus (B/New York/1121/2007) PA Wuhan spiny eel influenza virus	ANW79211 AHL92298 AVM87622	99%; 69.46% 99%; 48.58% 96%; 53.89%	0.0 0.0 0.0
Chum salmon influenza-like virus	Chum salmon (<i>Oncorhynchus keta</i>)	Gills	[47]	PB1 Influenza B virus (B/Iowa/14/2017) PB2 Influenza B virus (B/Memphis/5/93) PA Influenza B virus (B/Taiwan/45/2007)	QHI05420 AAU94860 ACO06009	100%; 69.50% 99%; 57.05% 98%; 51.88%	0.0 0.0 0.0
Cane toad influenza-like virus	Cane Toad (<i>Rhinella marina</i>)	Tissue unknown, Larval	[48]	PB1 Influenza D virus (bovine/Mexico/S7/2015) PB2 Influenza D virus (bovine/Kansas/14-22/2012) P3 Influenza D virus (bovine/Yamagata/10710/2016)	AMN87903 AIO11621 BBC14929	99% ; 77.03% 99%; 62.87% 100%; 62.66%	0.0 0.0 0.0
Ornate chorus frog influenza-like virus	Ornate chorus frog (<i>Microhyla fissipes</i>)	Larval, Lung	[50, 51]	PB1 Influenza D virus (bovine/Mississippi/C00046N/2014) PB2 Influenza D virus (swine/Italy/173287-4/2016) P3 Influenza D virus (D/bovine/Shandong/Y217/2014)	ALE66333 AON76692 AIE52099	98%; 81.91% 99%; 68.22% 99%; 63.78%	0.0 0.0 0.0