1 Identification of novel avian and mammalian deltaviruses provides new insights 2 into deltavirus evolution

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27

28 **Abstract**

29 Hepatitis delta virus (HDV) is a satellite virus that requires hepadnavirus envelope
30 proteins for its transmission. Although recent studies identified HDV-related deltaviruses
31 in certain animals, the evolution of deltaviruses, such as the origin of HDV and the
32 mechanism of its coevolution with its helper viruses, is unknown, mainly because of the
33 phylogenetic gaps among deltaviruses. Here we identified novel deltaviruses of passerine
34 birds, woodchucks, and white-tailed deer by extensive database searches and molecular
35 surveillance. Phylogenetic and molecular epidemiological analyses suggest that HDV
36 originated from mammalian deltaviruses and the past interspecies transmission of
37 mammalian and passerine deltaviruses. Further, metaviromic and experimental analyses
38 suggest that the satellite-helper relationship between HDV and hepadnavirus was
39 established after the divergence of the HDV lineage from non-HDV mammalian
40 deltaviruses. Our findings enhance our understanding of deltavirus evolution, diversity,
41 and transmission, indicating the importance of further surveillance for deltaviruses.

42 **Introduction**

43 Hepatitis delta virus (HDV) is the only member of the genus *Deltavirus* which is not
 44 assigned to a family (1). The HDV genome is an approximately 1.7-kb circular, negative
 45 single-stranded RNA, harboring a single open reading frame (ORF) encoding the small
 46 and large hepatitis delta antigens (S-HDAg, 24 kDa and L-HDAg, 27 kDa) that are
 47 translated from the same transcriptional unit via RNA-editing of the stop codon, which is
 48 catalyzed by the host protein ADAR1 (2-5). The 19 amino acid residue extension of the
 49 C-terminal region of L-HDAg contains a farnesylation site required to interact with
 50 helper virus envelope proteins (6). The genome structure of HDV is unique in that it has
 51 genomic and antigenomic ribozymes, which are essential for its replication (7, 8), and is
 52 highly self-complementarity, generating a rod-like structure (9-11). Although HDV can
 53 autonomously replicate, it requires an envelope protein of other “helper” viruses to
 54 produce infectious virions. Hepatitis B virus (HBV) (family *Hepadnaviridae*) provides
 55 the envelope proteins required for HDV transmission between humans (12).
 56 Approximately 15–20 million people worldwide are estimated to be infected with HDV
 57 among 350 million HBV carriers (13). Compared with mono-infection with HBV,
 58 coinfection of HDV and HBV accelerates the pathogenic effects of HBV, such as severe
 59 or fulminant hepatitis and progression to hepatocellular carcinoma, through unknown
 60 mechanisms (14).

61 The evolutionary origin of HDV presents an enigma. However, recent discoveries of
 62 deltaviruses of vertebrate and invertebrate species (15-18), significantly changed our
 63 understanding of deltavirus evolution. These non-HDV deltaviruses are distantly related
 64 to HDV but may share the same origin because of their similarly structured circular RNA
 65 genomes (approximately 1.7 kb), which encode DA_g-like proteins, possess ribozymes

66 sequences, and are highly self-complementary (15-18). These findings provide clues to
67 the mechanism of deltavirus evolution. For example, a recent study hypothesizes that
68 mammalian deltaviruses codiverged with their host mammalian species (18). However,
69 the few known deltaviruses are highly divergent (15-18). Therefore, the phylogenetic
70 gaps between the deltaviruses must be filled through the identification of putative novel
71 deltaviruses.

72 The discoveries of non-HDV deltaviruses provides insights into the relationships
73 between deltaviruses and their helper viruses. Recently identified non-HDV deltaviruses
74 likely do not coinfect with hepadnaviruses, suggesting the presence of other helper
75 viruses (15-18). This hypothesis is supported by absence of a large isoform of DA_g,
76 which is required for the interaction of HDV with the HBV envelope proteins, in rodent
77 deltavirus (18). Further, viral envelope proteins of reptarenavirus and hartmanivirus, but
78 not HBV, confer infectivity upon the snake deltavirus (19). These findings suggest that
79 hepadnaviruses do not serve as helper viruses for non-HDV deltaviruses and that the
80 deltavirus-hepadnavirus relationship is specific to the HDV lineage. However, the large
81 phylogenetic gap between HDV and the few other deltaviruses makes it difficult to assess
82 the hypothesis, raising the importance of further research.

83 In this study, to understand the evolution of deltaviruses, we analyzed publicly
84 available transcriptome data and found novel mammalian and avian deltaviruses. Our
85 phylogenetic analysis suggests that HDVs originated from non-HDV mammalian
86 deltaviruses and does not support the codiversification hypothesis of deltavirus and
87 mammalian evolution. Moreover, *in silico* and experimental analyses, together with
88 previous findings, suggest that the satellite-helper relationship between HDV and
89 hepadnavirus was established after the divergence of the novel non-HDV mammalian

90 deltaviruses and the HDV lineage. Further, we present evidence for recent interfamily
91 transmission of deltaviruses among passerine birds. Our findings therefore provide novel
92 insights into the evolution of deltaviruses.
93

94 **Results**

95 ***Identification of deltavirus-related sequences in avian and mammalian transcriptomes***

96 We first assembled 46,359 RNA-seq data of birds and mammals. Using the resultant
97 contigs as queries, we identified five deltavirus-related contigs in the SRA data of birds
98 and mammals, including the zebra finch (*Taeniopygia guttata*), common canary (*Serinus*
99 *canaria*), Gouldian finch (*Erythrura gouldiae*), Eastern woodchuck (*Marmota monax*),
100 and white-tailed deer (*Odocoileus virginianus*). We named the deltavirus-like sequences
101 *Taeniopygia guttata* deltavirus (tgDeV), *Serinus canaria*-associated deltavirus (scDeV),
102 *Erythrura gouldiae* deltavirus (egDeV), *Marmota monax* deltavirus (mmDeV), and
103 *Odocoileus virginianus* (ovDeV), respectively (Table 1).

104 The amino acid sequences translated from the contigs are 36.0%–66.7% identical to
105 those of the DAg proteins of known deltaviruses (Table 1, Supp Tables 1 and 2). The
106 tgDeV, mmDeV, and ovDeV contigs, which comprise approximately 1,700 nucleotides,
107 encode one ORF with a sequence similar to those of DAg genes of known deltaviruses
108 (Fig 1a, Table 1, Supp Tables 1 and 2). In contrast, the contigs scDeV and egDeV are 761
109 and 596 nucleotides in length, respectively (Fig. 1b, Supp Tables 1 and 2). Note that the
110 nucleotide sequences of tgDeV and egDeV are 97.7% identical, and we therefore
111 analyzed tgDeV instead of tgDeV and egDeV.

112

113 ***Genome structures of novel avian and mammalian deltaviruses***

114 The three contigs (tgDeV, mmDeV, and ovDeV) are almost identical in length to the
115 full-length genomes of known deltaviruses. We therefore checked for potential circularity
116 of the contigs. Dot-plot analyses revealed that each of both ends of these three contigs is
117 identical (Supp Fig. 1), suggesting that the contigs were derived from circular RNAs. We

118 further mapped the original RNA-seq data to the corresponding circularized contigs using
119 the Geneious mapper, revealing that some of the reads properly spanned the junctions
120 (data not shown), indicating that these contigs are derived from circular RNAs. Therefore,
121 we designated the resultant circular contigs of tgDeV, mmDeV, and ovDeV as full-length
122 novel deltavirus genomes (1,706, 1,712, and 1,690 nucleotides, respectively) (Fig. 1a).
123 These novel genomes are characterized by high self-complementarity, genomic and
124 antigenomic ribozymes, and poly(A) signals, which are conserved among known
125 deltaviral genomes (Fig. 1 and Supp Table 2) (15-18). Further, the predicted secondary
126 structures of the ribozymes are highly similar to those of HDV as well as those of other
127 deltaviruses (Supp Fig. 2).

128

129 ***Characterization of DAg proteins encoded by the novel deltaviruses***

130 We next characterized the putative DAg proteins encoded by the novel deltaviruses. Most
131 of their biochemical features, biologically relevant amino acid residues, and functional
132 domains (15-18) are conserved among the DAg proteins (Fig. 2a). The isoelectric points
133 of DAg proteins from the novel deltaviruses range from 10.35 to 10.63 (Supp Table 2),
134 which are nearly identical to those of known deltaviruses. All the post-translational
135 modification sites in HDAg are conserved among those of all DAg proteins of the novel
136 deltaviruses, except for the serine phosphorylation site on scDeV-DAg (Fig. 2a). The
137 NLS is conserved among the DAg proteins, although location of the predicted NLS of
138 scDeV DAg protein differs (Fig. 2a).

139 We next investigated whether the novel deltaviruses utilize A-to-I RNA-editing. To
140 answer this question, we mapped short reads of the SRA data, which we initially used to
141 detect the deltaviruses, to identify the nucleotide variations among the stop codons. We

found a potential RNA-editing site within the stop codon of the ovDeV-DAG gene, in which there was 0.4% nucleotide variation (5 of 1160 reads) at the second nucleotide position of the stop codon (UAG), all of which were G instead of the consensus nucleotide A (Fig. 2b). The quality scores of the five G variants ranged from 35 to 41 (Supp Fig. 3), which likely exclude the possibility of a sequencing error. This variation may be explained by A-to-I editing by ADAR1, as known for HDV (2). However, possible RNA-editing generates a protein two amino acid residues longer because of a stop codon immediately downstream (Fig. 2c). Further, the C-terminal farnesylation motif (CXXQ) required for the interaction with hepadnaviral envelope proteins (6) was absent from the longer product. These observations suggest that even if RNA-editing occurs, the resultant gene product does not contribute to the interaction with hepadnaviral envelope proteins. Further, we were unable to identify nucleotide variations of the mapped reads at the stop codons in the genomes of tgDeV and mmDeV (data not shown).

The novel deltaviruses potentially replicate in their hosts

To determine whether the novel deltaviruses potentially replicate in their respective, putative host species, we evaluated the mapping pattern of viral reads described above. We found that the read depths of the predicted transcribed regions (the DAG coding regions to poly-A signals) were much greater than those of the other genomic regions (Fig. 3), indicating that most viral reads were derived from viral mRNAs. These findings suggest that the novel deltaviruses replicate in their hosts.

The mapping pattern on tgDeV differed slightly from the others. Specifically, although the read depth of the DAG ORF region was higher, the reads represented only 80% of the ORF (Fig. 3a). This trend was apparent in another tgDeV-positive RNA-seq

166 data (Supp Fig. 4). However, it is not clear whether this is attributed to an artifact or
167 actually reflects the transcription pattern of tgDeV.

168

169 ***Transmission of tgDeV- and tgDeV-like viruses among passerine birds***

170 We next investigated whether the novel deltaviruses are transmitted among animal
171 populations. We first analyzed tgDeV infections in birds using RNA-seq data (Table 2
172 and Supp Table 3). Among 6453 SRA data, tgDeV-derived reads were identified in 34
173 SRAs, including the SRAs in which tgDeV and egDeV were initially detected. The 34
174 tgDeV-positive SRA data were obtained from tissues such as blood, kidney, and muscles,
175 suggesting broad tropism and viremia, or systemic infection, or both, with tgDeV.

176 Further, tgDeV sequences were detected in several bird species such as the
177 black-headed bunting (*Emberiza melanocephala*) and yellow-bellied tit (*Pardaliparus*
178 *venustulus*). All tgDeV-positive bird species belong to the order Passeriformes. These
179 tgDeV-positive SRA data are included in the nine BioProjects deposited by independent
180 researchers, and thus the birds were likely from different sources. Further, the
181 tgDeV-positive sample in SRR9899549 (BioSample accession: SAMN12493457) is
182 derived from a black-headed bunting caught in the wild. These data suggest that tgDeV
183 (or tgDeV-like viruses) circulate among diverse passerine birds, even in the wild.

184 During the above analysis, we found that SRA data from the yellow-bellied tit
185 (SRR7244693 and SRR7244695–SRR7244698) contain many reads mapped to the
186 tgDeV genome. Therefore, we extracted the mapped reads of SRA data and performed *de*
187 *novo* assembly. We obtained a 1707-nt circular complete genome sequence, which we
188 designated pvDeV. The pvDeV nucleotide sequence is 98.2% identical to that of tgDeV,
189 and the properties of its DA_g protein sequence are similar to those of tgDeV DA_g (Supp

190 Fig. 5).

191 We next employed real-time RT-PCR to further evaluate potential deltavirus
192 infections of passerine birds. We analyzed 30 and 5 whole-blood samples from zebra and
193 Bengalese finches (*Lonchura striata* var. *domestica*), respectively, and found that one
194 Bengalese finch was positive for real-time RT-PCR test. To exclude the possibility of
195 contamination of a plasmid used as a control for real-time PCR, we performed RT-PCR
196 using a primer set that distinguishes viral from plasmid amplicons (Figs. 4a and b). We
197 obtained a band of the expected size only from the cDNA sample (Fig. 4c), revealing that
198 the bird was truly positive for a tgDeV-like virus. Therefore, we named this virus lsDeV,
199 and further analysis revealed that its full-length genome nucleotide sequence (1708 nt) is
200 98.2% and 98.4% identical to those of tgDeV and pvDeV, respectively. Moreover, its
201 genome features are almost identical to those of tgDeV and pvDeV (Supp Fig. 5).

202

203 ***Evidence for recent interfamily transmission of deltaviruses among passerine birds***

204 We found that the sequence similarities among the passerine deltaviruses (tgDeV, pvDeV,
205 and ls DeV) (Fig. 4d) were not consistent with evolutionary codivergence. According to
206 the TimeTree (20), deltavirus-positive passerine birds diverged approximately 44 million
207 years ago (Fig. 4e and Supp Fig. 6). Considering the rapid evolutionary rates of HDVs
208 (approximately 10^{-3} substitutions per site per year) (21-23), it is unlikely that these
209 viruses codiverged with their hosts. Most likely, interfamily transmission occurred
210 relatively recently among passerine birds.

211

212 ***Transmission of mmDeV among woodchucks***

213 We similarly analyzed mmDeV infections using SRA data for the order Rodentia, other

214 than mice (*Mus musculus*) and rats (*Rattus norvegicus*). Our analysis of 4776 SRA
215 datasets detected mmDeV reads in 20 SRA data of seven woodchucks (Table 2 and Supp
216 Table 3). Although these mmDeV-positive SRA data were contributed by the same
217 research group, the animals were apparently obtained at different times (24, 25),
218 suggesting that mmDeV was transmitted among woodchucks. The mmDeV-positive SRA
219 data are derived from samples of liver or peripheral blood mononuclear cells.

220 We next used real-time RT-PCR to analyze 81 woodchuck samples (liver, n= 43;
221 serum, n = 38). However, mmDeV was undetectable (data not shown), which may be
222 explained by the absence of mmDeV infection or clearance, low level of infection, or
223 both.

224

225 ***No evidence of transmission of other deltaviruses***

226 We next focused on ovDeV and scDeV sequences of ruminant animals and passerine
227 birds, respectively (Table 2 and Supp Table 3). We detected ovDeV-derived reads only in
228 five SRA data. The SRA data were obtained from brain, muscle, testis, pedicles, and
229 antlers, suggesting systemic infection, viremia, or both. However, we were unable to
230 determine if these samples were derived from multiple individuals. We detected
231 scDeV-derived reads only from the SRA data in which the virus was initially detected. We
232 therefore were unable to provide evidence for the transmission of ovDeV and scDeV in
233 their host animals.

234

235 ***Phylogenetic relationships among deltaviruses***

236 To decipher the evolutionary relationships among deltaviruses, we conducted a
237 phylogenetic analysis using known and the novel deltavirus sequences discovered here.

238 We did not include sequences of recently identified fish, toad, newt, termite, and
239 duck-associated deltaviruses because they share very low amino acid sequence identities
240 with the novel deltaviruses as well as with HDVs (Fig. 5a), which may reduce the
241 accuracy of tree (16). We further excluded scDeV for this reason, and we did not include
242 tgDeV-like viruses, because their sequences are nearly identical to that of tgDeV. The
243 reconstructed tree shows that the newly identified tgDeV forms a strongly supported
244 cluster with snake DeV and rodent DeV, although they are distantly related to each other
245 (Fig. 5b). Note that mmDeV and ovDeV are more closely related to HDVs than the other
246 deltaviruses.

247

248 *Candidate helper viruses*

249 To gain insights into helper viruses of the novel deltaviruses, we first analyzed the
250 coexisting viruses in the SRA data using BLASTx. Note that we omitted experimental
251 woodchuck hepatitis virus (WHV) infections associated with the mmDeV-positive
252 woodchuck-derived SRA data (SRR2136864 to SRR2136999). We also excluded viruses
253 that infect invertebrates and endogenous retroviruses as well. These analyses reveal that
254 polyomavirus, bornavirus, and circovirus sequences are present in the deltavirus-positive
255 SRA data of passerine birds (Table 3 and Supp Table 4). Further, we detected contigs with
256 98%–100% identities to human viruses (human mastadenovirus or mammalian
257 rubulavirus 5) (Supp Table 4), although these may represent contamination, index
258 hopping, or both. Among these viruses, only the genome of bornavirus encodes an
259 envelope protein. Note that scDeV and bornavirus-positive SRA data was obtained from
260 pooled samples (SAMN04260514), and we therefore were unable to determine whether
261 scDeV and canary bornavirus 3 infected the same individual.

262 We next cross-referenced the mmDeV reads and the metadata, which also provide
263 insights into the mmDeV helper virus. Among 20 mmDeV-positive SRA data, 18 were
264 obtained from animals experimentally infected with the hepadnavirus WHV, which was
265 experimentally shown to serve as a helper virus for HDV (26, 27). However, the other
266 two SRA data (SRR437934 and SRR437938) were derived from animals negative for
267 antibodies against WHV as well as WHV DNA (24). These observations suggest that
268 mmDeV was transmitted to the two animals without WHV and that WHV therefore was
269 not the helper virus for mmDeV that infected these two individuals.

270

271 ***Replication of tgDeV and mmDeV in human and woodchuck cell lines***

272 To investigate whether the novel deltavirus sequences are replicable or not, we performed
273 transfection-based assays in cell culture systems. We constructed plasmid expression
274 vectors harboring the minus-strand genome of the tgDeV or mmDeV dimer sequence
275 under the transcriptional control of the CMV promoter (see Materials and Methods). We
276 first determined if the replication initiated by transfecting these plasmids. These plasmids
277 express the minus-strand genome and therefore DAg protein is expressed if the viral
278 genome replicates (19). We transfected the plasmid vectors into Huh7 human hepatic
279 cells and WCH-17 woodchuck hepatic cells and used western blotting (Figs. 6a and b)
280 and immunofluorescence assay (IFA) (Figs. 6c–f) to detect the expression of DAg
281 proteins. Western blotting detected the expected bands (approximately 22 kDa) only in
282 transfected cells (Figs. 6a and b). Note that a single specific band was detected in lysates
283 prepared from each cell type, suggesting that tgDeV and mmDeV expressed only one
284 DAg isoform. Consistent with the above results, specific signals were observed only in
285 the transfected cells in IFA (Figs. 6c and d, red signals). Together, these data suggest that

286 the tgDeV and mmDeV initiated replication from the constructed plasmids in the cell
287 culture system.

288 The DAG proteins predominantly localized to the nucleus 2 days post-transfection
289 (Figs. 6e and f). Interestingly, large viral speckles were observed in the nucleus, similar to
290 those detected in cells infected with HDV (28, 29). These results suggest that tgDeV and
291 mmDeV employ a nuclear replication strategy similar to that used by HDV.

292

293 ***HBV envelope proteins do not contribute to the production of infectious tgDeV and***
294 ***mmDeV***

295 As described in the above section “*Candidate helper viruses*”, there is no evidence of
296 coinfections of hepadnaviruses with tgDeV or mmDeV. However, this does not
297 necessarily mean hepadnaviruses do not serve as helper viruses for the novel deltaviruses.
298 To determine whether tgDeV or mmDeV utilize the HBV envelope proteins (HBs), we
299 transfected the deltavirus expression plasmids together with an HBs expression vector or
300 the cognate empty vector into Huh7 cells. The culture supernatants were incubated with
301 HepG2-NTCP cells, which are susceptible to HBs-dependent HDV infection (30, 31).
302 HDV served as a control to monitor HBs-dependent virus release and subsequent cell
303 entry. Viral RNA was undetected in supernatants of cells that did not express HBs (Fig.
304 7a). In contrast, cotransfection of the HBs plasmid released large amounts of HDV RNA
305 into the supernatant, consistent with published data (32), whereas tgDeV or mmDeV
306 RNA was undetectable (Fig. 7a).

307 We next measured the amounts of viral RNA and detected DAG protein in
308 HepG2-NTCP cells 7 days after incubation with the supernatants. HDV RNA and DAG
309 protein were highly expressed in the infected cells (Fig. 7b and c). HDV infection was

310 inhibited by the preS1 peptide (Myrcludex B), which was shown to inhibit
311 HBs-dependent HDV infection (33). These indicate that HDV infection is indeed
312 mediated by the HBs. On the other hand, tgDeV and mmDeV RNA or DAg protein was
313 undetectable, suggesting that HBs do not contribute to the production of infectious tgDeV
314 or mmDeV.

315 Discussion

316 Important aspects of the evolution of deltaviruses are unknown, such as the origin of
 317 HDV and the coevolution of deltaviruses and their helper viruses, mainly because few
 318 deltaviruses are known, and they are highly genetically divergent (15-18). Therefore, the
 319 resulting large gaps in the deltavirus phylogenetic tree create a formidable obstacle to
 320 understanding deltavirus evolution. Here we identified five complete genomes of novel
 321 deltaviruses from birds and mammals (Fig. 1 and Supp Fig. 5), which partially fill these
 322 phylogenetic gaps (Fig. 5b). Moreover, our present findings reveal that the evolution of
 323 deltaviruses is much more complicated than previously thought. For example, one
 324 hypothesis states that mammalian deltaviruses codiverged with their mammalian hosts
 325 (18). However, our phylogenetic analysis shows that the tree topology of mammalian
 326 deltaviruses is incongruent with their hosts'. For example, ovDeV, which we detected in
 327 deer, is most closely related to human HDV (Fig. 5b). Further, the distantly related
 328 mmDeV and rodent DeV (detected in *Proechimys semispinosus* (18)) were detected in
 329 rodent species. These data suggest that deltaviruses were transmitted among mammalian
 330 species and did not always codiverge with their hosts. Moreover, we discovered recent
 331 interfamily transmission of passerine deltaviruses (tgDeV and its relatives) (Fig. 4e).
 332 Therefore, avian and mammalian deltaviruses may have, at least partially, evolved by
 333 interspecies transmission.

334 Our present phylogenetic analysis also gives insights into the origin of human HDVs.
 335 As described above, ovDeV and mmDeV are close relatives of human HDVs, suggesting
 336 that HDVs arose from other mammalian deltaviruses. Recent studies on the phylogeny of
 337 bat deltaviruses support our findings and conclusions (34, 35) (Supp Fig. 7). However, we
 338 were unable to exclude the possibility of infection of animal lineages apart from

339 mammals with unknown deltaviruses phylogenetically located between those viral
340 lineages. Further investigations are required for a better understanding of the deltavirus
341 evolution.

342 There is a paucity of knowledge about helper viruses for non-HDV deltaviruses,
343 other than the snake deltavirus (19), although evidence indicates that hepadnaviruses may
344 not serve as helper viruses for the novel deltaviruses discovered here, as suggested for
345 other non-HDV deltaviruses (15-18). Here we only detected bornavirus, circovirus, and
346 polyomavirus, but not hepadnavirus sequences in association with deltavirus-positive
347 SRA data (Table 3). Further, mmDeV was detected in two woodchuck individuals that
348 were demonstrated to be negative for WHV (Table 2 and Supp Table 3). Moreover, we
349 found that HBs did not contribute to the formation of infectious tgDeV and mmDeV in
350 cell culture experiments. These observations suggest that hepadnaviruses do not serve as
351 helper viruses for the novel non-HDV deltaviruses detected here. Further, we were
352 unable to demonstrate that the deltaviruses identified here express proteins similar to the
353 L-HDAg protein (Figs. 6a and b), which is expressed via RNA-editing and is essential for
354 HDV to interact with HBs (2, 37). Although RNA-editing may alter the stop codon of
355 ovDeV DAg, this does not lead to the expression of a large isoform of DAg (L-DAg)
356 protein containing a farnesylation site (Figs. 2b and c). The lack of L-DAg expression
357 was also observed in rodent deltaviruses (18). Therefore, L-DAg expression phenotype
358 may have been acquired after the divergence of ovDeV and the HDV lineage (Fig. 5b).

359 Among the coexisting viruses, only bornavirus produces an envelope glycoprotein
360 (G protein), which might be used by non-HDV deltaviruses to produce infectious virions.
361 Indeed, snake deltavirus utilizes the envelope proteins of reptarenaviruses and
362 hartmaniviruses to produce infectious particles (19). Further, HDV forms infectious

363 virions using envelope proteins *in vitro* of RNA viruses such as vesiculovirus and
364 hepacivirus (38). Therefore, the bornavirus G protein might envelop non-HDV
365 deltaviruses.

366 In contrast, the coexisting viruses, circoviruses and polyomaviruses, are
367 nonenveloped. Therefore, it is unlikely that these viruses can serve as helper viruses for
368 the deltaviruses. However, we cannot exclude the possibility that these viral capsid
369 proteins might contribute to the transmissibility of deltaviruses through unknown
370 mechanisms. Additionally, virus-derived sequences in host genomes, called endogenous
371 viral elements (EVEs), might mediate the formation of infectious particles. Here detected
372 the expression of retroviral EVEs in certain deltavirus-positive SRA data (data not
373 shown). Although HDVs do not use retroviral envelope proteins (38), non-HDV
374 deltaviruses might utilize strategies distinct from those employed by HDVs. Alternatively,
375 non-HDV deltaviruses may not require helper viruses and utilize extracellular vesicles for
376 transmission. Further biological experiments, together with molecular surveillance, are
377 therefore required to understand the satellite-helper relationships of deltaviruses.

378 Here we show that the sequences of tgDeV and tgDeV-like viruses, such as pvDeV
379 and lsDeV, are relatively closely related to known vertebrate deltaviruses (Fig. 5b).
380 Although a previous study found a deltavirus from duck, this duck-associated virus was
381 detected in oropharyngeal/cloacal swabs and is distantly related to vertebrate deltaviruses,
382 suggesting the possibility of its dietary origin (15, 18). This may be true for scDeV
383 studied here. scDeV was detected in skin (Table 1). Although scDeV was excluded from
384 our phylogenetic analysis, the amino acid identities between the DAg protein of scDeV
385 with other vertebrate deltaviruses range from 32.7%–39.5% (Fig. 5a). Therefore, scDeV
386 may be derived from contaminants, which should be addressed in the future. In contrast,

387 tgDeV and tgDeV-like viruses were detected in tissues such as the spleen and muscles
388 (Table 2), suggesting that tgDeV and tgDeV-like viruses are authentic avian deltaviruses.

389 Here we show that certain novel deltaviruses are transmitted among animal
390 populations (Table 2 and Supp Table 3). Note that few reads were mapped to the virus
391 genomes in some SRA data, which may be attributed to index hopping (39-43) from SRA
392 data containing numerous deltavirus-derived reads. Therefore, these data should be
393 interpreted with caution. Nevertheless, our conclusions are not affected, because they are
394 supported by robust data (Table 2). For data in which index hopping has possibly
395 occurred, further analyses are needed to confirm deltavirus infections.

396 Our present analysis provides virological insights into important characteristics of
397 deltavirus infections, such as tissue and host tropism. For example, infections with tgDeV
398 (and tgDeV-like viruses), mmDeV, and ovDeV were not limited to the liver and were
399 detected in at least two different tissues (Table 2). These observations are consistent with
400 those of previous studies that non-HDV deltaviruses in multiple organs and blood and
401 that they replicate in numerous cell types (18, 19). Therefore non-HDV deltaviruses may
402 infect diverse tissues and cause systemic infection, viremia, or both. Further, tgDeV and
403 mmDeV replicated in human and woodchuck cells (Fig. 6), which is consistent with the
404 ability of the snake deltavirus to replicate in mammalian cells (19). These observations
405 suggest that the host range of deltaviruses is broad and that the helper viruses of
406 non-HDV deltaviruses may be the determinants of host range.

407 Our analyses further suggest that tgDeV and mmDeV are sensitive to host immune
408 responses. We cross-referenced tgDeV reads and metadata and made an intriguing
409 finding that may contribute to the virus-host interaction. BioProject PRJNA297576
410 contains 12 RNA-seq data for six zebra finches (44). Interestingly, the tgDeV reads were

almost exclusively detected in birds treated with testosterone vs the controls (Supp Fig. 8a and Supp Table 5). Therefore, the immunosuppressive effects of testosterone (45) may increase the transcription or replication of tgDeV, or both, to enable detection using RNA-seq. Further, when we cross-referenced the mmDeV reads and the metadata, we found that 18 of 20 mmDeV-positive SRA data derived from five individuals were acquired through an experiment lasting 27 weeks (PRJNA291589) (25). Among 18 SRA data, those of one individual (ID 1008) provide insights into mmDeV infection as follows: At first (–3 weeks), mapped reads were not detected, although the proportion of mapped reads were highest at one week and then drastically decreased (Supp Fig. 8b and Supp Table 6). Interestingly, a previous study suggested that the host’s immune response can clear rodent deltaviruses (18). Our present observations together with this previous finding, suggest that the host immune response suppresses and then clear deltavirus infections. This may explain the low prevalence of RT-PCR-positive samples of woodchucks and passerine birds as described in the Results (sections “*Circulation of tgDeV and tgDeV-like viruses among passerine birds*” and “*Circulation of mmDeV in woodchucks*”). Note that latent or low levels of persistent deltavirus infections may occur. Indeed, snake deltavirus establishes a persistent infection in a cell culture system (19). Therefore, deltaviruses might persistently infect host cells with a low level of virus replication, and some stimulations, such as immunosuppression, may trigger robust virus replication. Further studies are therefore required to understand the interactions between deltaviruses and their hosts.

Together, our present data contribute to a deeper understanding of the evolution of deltaviruses and suggest the presence of undiscovered deltaviruses that infect diverse animal species. Further investigations will provide further insights into deltavirus

435 evolution.

436

437 **Materials and methods**

438 *Detection of deltaviruses from publicly available transcriptome data*

439 Paired-end, RNA-seq data from birds and mammals were downloaded from NCBI SRA
 440 (46). The SRA accession numbers used in this study are listed in Supplementary material.
 441 The downloaded SRA files were dumped using pfastq-dump (DOI:
 442 10.5281/zenodo.2590842; <https://github.com/inutano/pfastq-dump>), and then
 443 preprocessed using fastp 0.20.0 (47). If genome data of either the same species or the
 444 same genus were available, the preprocessed reads were mapped to the corresponding
 445 genome sequences (the genome information is available upon request) by HISAT2 2.1.0
 446 (48), and then unmapped paired-end reads were extracted using SAMtools 1.9 (49) and
 447 Picard 2.20.4 (<http://broadinstitute.github.io/picard/>). The extracted unmapped reads
 448 were used for *de novo* assembly. If genome data were unavailable, the preprocessed reads
 449 were directly used for *de novo* assembly. *De novo* assembly was conducted using SPAdes
 450 (50) and/or metaSPAdes (51) 3.13.0 with k-mer of 21, 33, 55, 77, and 99. The resultant
 451 contigs were clustered by cd-hit-est 4.8.1 (52, 53) with a threshold of 0.95. Finally, the
 452 clustered contigs ≥ 500 nt were extracted by SeqKit 0.9.0 (54), and they were used for the
 453 downstream analyses.

454 Two-step sequence similarity searches were performed to identify RNA virus-like
 455 sequences. First, BLASTx searches were performed against a custom database for RNA
 456 viruses using the obtained contigs as queries employing BLAST+ 2.9.0 (55) with the
 457 following options: -word_size 2, -evalue $1e^{-3}$, max_target_seqs 1. The custom database of
 458 RNA viruses consisted of clustered sequences (by cd-hit 4.8.1 with a threshold of 0.98)
 459 from viruses of the realm *Riboviria* in the NCBI GenBank (the sequences were
 460 downloaded on June 2, 2019) (46). Next, the query sequences with viral hits were

461 subjected to second BLASTx analyses, which were performed against the NCBI nr
462 database. Finally, the second blast hits with the best hit against deltaviruses were regarded
463 as deltavirus-like agents, and they were used for detailed analyses.

464

465 *Confirmation of circularities of deltavirus contigs*

466 Self dot-plot analyses of linear deltavirus contigs were conducted using the YASS online
467 web server (29). Based on the analysis, the contigs were manually circularized using
468 Geneious 11.1.5 (<https://www.geneious.com>). Further confirmation of the circularities of
469 deltavirus contigs was obtained by mapping short reads to circular deltavirus contigs
470 using Geneious software as follows. The reads used for the *de novo* assembly were first
471 imported to Geneious, after which they were mapped to the circular contigs using the
472 Geneious mapper. The mapped reads across the circularized boundaries were confirmed
473 manually.

474

475 *Detection of possible RNA-editing sites at stop codons of DAg genes*

476 We used the mapped reads obtained by the analyses described above to detect possible
477 RNA-editing at the stop codons of DAg genes of deltaviruses. We analyzed the
478 nucleotide variations (presence of variations, variant nucleotide(s), and variant
479 frequency) of mapped reads at each of the stop codons of newly identified deltaviruses
480 using the “Find Variation/SNPs” function in Geneious. We used a custom Python script to
481 visualize base quality scores of the NGS reads mapped at the second nucleotide of the
482 stop codon genome. The codes are available at following URL:
483 https://github.com/shohei-kojima/iwamoto_et_al_2020.

484

485 *Sequence characterization*

486 DAg ORFs were detected by the “Find ORFs” function in Geneious with a threshold of
487 500 nucleotides. Poly(A) signals were manually detected. Putative ribozyme sequences
488 were identified using nucleotide sequence alignment with other deltaviruses. Ribozyme
489 structures were first inferred using the TT2NE webserver (56), and the obtained data were
490 then visualized using the PsudoViewer3 web server (57). We used the visualized data as
491 guides to draw ribozyme structures.

492 The self-complementarities of deltavirus-like contigs were analyzed using the Mfold
493 web server (58). Coiled-coil domains and nuclear export signals (NLSs) were predicted
494 using DeepCoil (59) and NLS mapper
495 (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) web servers,
496 respectively.

497

498 *Short read mapping for detection of deltavirus infection*

499 To detect deltavirus-derived reads in publicly available RNA-seq data, short reads were
500 mapped to deltavirus genomes and then the numbers of mapped reads were counted as
501 follows. SRA files were downloaded from NCBI, dumped, and preprocessed following
502 the procedure described above. The preprocessed reads were then mapped to linearized
503 deltavirus contigs by HISAT2 with the default setting. SAM tools were used to extract the
504 mapped BAM files from the resultant BAM files, and the mapped read numbers were
505 counted using BamTools 2.5.1 (60).

506

507 *Recovery of a deltavirus genome from RNA-seq data of *Pardaliparus venustulus**

508 Mapped reads obtained from SRR7244693, SRR7244695, SRR7244696, SRR7244697,

509 and SRR7244698 in the above analysis (section *Short read mapping for detection of*
510 *deltavirus infection*) were extracted by Geneious. All the extracted reads were
511 co-assembled using Geneious Assembler with the circular contig assembly function. The
512 obtained circular contigs were characterized as described previously.

513

514 *Animals and samples*

515 Zebra finches (n = 30) and Bengalese finches (n = 5) were obtained from breeding
516 colonies at Wada lab, Hokkaido University. The founder birds were originally obtained
517 from local breeders in Japan. Five to twelve birds were kept together in cages in an aviary
518 and were exposed to a 13:11 light-dark cycle. Blood samples were collected from the
519 wing vein using 30 G × 8 mm syringe needles (Becton Dickinson; Franklin Lakes, NJ,
520 USA). Each blood sample was diluted 1.5 times with PBS, frozen immediately on dry ice
521 after collection, and maintained at −80°C until further requirement. These experiments
522 were conducted under the guidelines and with the approval of the Committee on Animal
523 Experiments of Hokkaido University. These guidelines are based on the national
524 regulations for animal welfare in Japan (Law for the Humane Treatment and
525 Management of Animals with partial amendment No.105, 2011).

526 Woodchucks (*Marmota monax*) were purchased from Northeastern Wildlife
527 (Harrison, ID, USA) and kept at the Laboratory Animal Center, National Taiwan
528 University College of Medicine. At three days of age, the animal supplier infected
529 captive-born woodchucks with WHV from the same infectious pool. Wild-caught
530 woodchucks were infected naturally and live trapped. Serum samples were collected
531 from the woodchucks periodically via the femoral vein by means of venipuncture. Liver
532 tissues of woodchucks were obtained at autopsy, snap-frozen in liquid nitrogen, and

533 stored at -80°C until RNA extraction. This study used liver tissues from 10 wild-caught
534 and 33 captive-born woodchucks and serum samples from 33 wild-caught and five
535 captive-born woodchucks. In this study, all the experimental procedures involving
536 woodchucks were performed under protocols approved by the Institutional Animal Care
537 and Use Committee of National Taiwan University College of Medicine.

538

539 *Real-time and Endpoint RT-PCR detection of deltaviruses from animal specimens*

540 Total RNAs were isolated from the whole blood samples from zebra finches and serum
541 samples from woodchucks using Quick RNA Viral Kit (Zymo Research; Irvine, CA,
542 USA). The obtained RNA samples were stored at -80°C until further requirement. Total
543 RNAs were also extracted from 50 mg of the woodchuck liver tissues using either Trizol
544 (Thermo Fisher Scientific; Waltham, MA, USA) or ToTALLY RNA kit (Thermo Fisher
545 Scientific; Waltham, MA, USA) according to the manufacturers' instructions.

546 The obtained RNA was reverse-transcribed into cDNA using ReverTra Ace qPCR
547 RT Master Mix (TOYOBO; Osaka, Japan), and these were used as templates for real-time
548 PCR analyses. Real-time PCR was performed with KOD SYBR qPCR Mix (TOYOBO)
549 and primers (Supp Table 7) using the CFX Connect Real-Time PCR Detection System
550 (Bio-Rad; Hercules, CA, USA) according to the manufacturer's instructions. The
551 real-time PCR systems for mmDeV and tgDeV were validated using
552 pcDNA3-mmDeV(-) and pcDNA3-tgDeV(-) monomer, respectively, as controls.

553 End-point RT-PCR was also performed to confirm deltavirus infections. PCR was
554 performed with Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific) using
555 the above-described cDNAs and primers listed in Supp Table 7. The PCR products were
556 analyzed by agarose gel electrophoresis. The obtained PCR products were purified and

557 sequenced by Sangar sequencing in FASMAC (Atsugi, Japan).

558

559 *Determination of a full genome sequence of deltavirus in passerine birds*

560 To determine the full genome sequence of detected deltaviruses, the cDNA obtained in

561 the section “*Realtime and Endpoint RT-PCR detection of deltaviruses from animal*

562 *specimens*” was amplified using illustra GenomiPhi V2 Kit (GE healthcare; Chicago, IL,

563 USA). The amplified DNA was then purified with innuPREP PCRpure Kit (Analytik

564 Jena: Jena, Germany). PCR was performed with Phusion Hot Start II DNA Polymerase

565 using the primers listed in Supp Table 7. The PCR products were analyzed using agarose

566 gel electrophoreses. When single bands were observed, the amplicon was purified with

567 innuPREP PCRpure Kit. When several bands were detected, bands of the expected sizes

568 were extracted and purified using Zymoclean Gel DNA Recovery Kit (Zymo Research).

569 The purified amplicons were sequenced in FASMAC (Atsugi, Japan).

570

571 *Phylogenetic analysis*

572 Deduced amino acid sequences of DAg proteins were used to infer the phylogenetic

573 relationship between deltaviruses. Multiple alignment was performed by MAFFT 7.427

574 using the E-INS-i algorithms (61), and ambiguously aligned regions were then removed

575 using trimAl 1.2rev59 with the --strict option (62). The phylogenetic relationship was

576 inferred by the maximum likelihood method using RAxML Next Generation v. 0.9.0 (63).

577 The LG+G model, which showed the lowest BIC by prottest3 3.4.2 (64), was used. The

578 reliability of the tree was assessed by 1,000 bootstrap resampling using the transfer

579 bootstrap expectation method (65). The alignment file is available in Supporting

580 materials.

581

582 *Detection of co-infected viruses*

583 To identify co-infected viruses in deltavirus-positive SRAs, a three-step BLASTx search
584 was performed. First, BLASTx searches were performed against a custom database,
585 including RefSeq protein sequences from viruses using the assembled contigs (see the
586 subsection *Detection of deltaviruses from publicly available transcriptome data*) as
587 queries. The custom database was prepared as follows. Virus-derived protein sequences
588 in the RefSeq protein database (46) were downloaded on July 17, 2020, and were
589 clustered by cd-hit 4.8.1 (threshold = 0.9). Then, sequences of more than 100 amino acid
590 residues were extracted using SeqKit 0.10.1 and these were used as a BLAST database.
591 The first BLAST hits were extracted, which were used for the second BLASTx analysis.
592 The second BLASTx analysis was performed against the NCBI RefSeq protein database.
593 The BLAST hits with the best hit to viral sequences were extracted and used for the final
594 BLASTx searches. The final BLASTx searches were performed against the NCBI nr
595 database. The BLAST hits with the best hit to viral sequences were extracted and
596 analyzed manually.

597

598 *Cell culture*

599 HepG2-NTCP cells were cultured with Dulbecco's modified Eagle's medium
600 (DMEM)/F-12 + GlutaMax (Thermo Fisher Scientific) supplemented with 10 mM
601 HEPES (Sigma Aldrich; St. Louis, MO, USA), 100 unit/ml penicillin (Meiji; Tokyo,
602 Japan), 100 mg/ml streptomycin (Meiji), 10% FBS (Sigma Aldrich), 5 µg/ml insulin
603 (Wako; Tokyo, Japan) and 400 g/ml G418 (Nacalai tesque). Huh7 and WCH-17 cells
604 were maintained in DMEM (Wako) containing 10% FBS (Sigma Aldrich), 100 unit/ml

605 penicillin (Meiji), 100 mg/ml streptomycin (Meiji), 100 mM nonessential amino acids
606 (Thermo Fisher Scientific), 1 mM sodium pyruvate (Sigma Aldrich), and 10 mM HEPES
607 (Sigma Aldrich).

608

609 *Antibody production*

610 The peptides corresponding to 65 to 78 aa (DSSSPRKRRKGEGG) of tgDeV DA_g and
611 174 to 187 aa (ESPYSRRGEGLDIR) of mmDeV DA_g conjugated with cysteine at N
612 terminus were synthesized. Each of the peptides was injected into mice, and antisera were
613 obtained from the mice at 42 days after the peptide injections. Each of the antisera was
614 affinity-purified using the corresponding peptide. The whole procedure was performed in
615 SCRUM (Tokyo, Japan).

616

617 *Rescue of mmDeV and tgDeV*

618 The DNA of negative-strand genomes of mmDeV and tgDeV was synthesized in
619 GenScript Japan (Tokyo, Japan). The synthesized DNAs were inserted into the KpnI
620 -XbaI site of the pcDNA3 vector, designated as pcDNA3-mmDeV(-) monomer and
621 pcDNA-tgDeV (-) monomer. In addition, tandem sequences of mmDeV and tgDeV
622 genome were inserted into the pcDNA3 vector, which were named pcDNA3-mmDeV(-)
623 dimer and pcDNA-tgDeV (-) dimer, respectively. To rescue these viruses,
624 pcDNA3-mmDeV(-) dimer or pcDNA-tgDeV (-) dimer was transfected into Huh7 and
625 WCH-17 cells using Lipofectamine 3000 and Lipofectamine 2000 (Thermo Fisher
626 Scientific), respectively, according to the manufacturer's instructions. The transfected
627 cells were cultured for 48 h and were used for western blotting, IFA to verify DA_g protein
628 expression.

629

630 *Western blotting*

631 Cells were lysed with SDS sample buffer [100 mM Tris-HCl (pH 6.8) (Sigma Aldrich),
632 4% SDS (Nippon gene; Tokyo, Japan), 20% glycerol (Nacalai tesque), 10%
633 2-mercaptoethanol (Wako)]. The cell lysates were subjected to SDS-PAGE and
634 transferred onto polyvinylidene difluoride membranes (Merck Millipore; Darmstadt,
635 Germany). After blocking the membranes with 5% skim milk (Morinaga; Tokyo, Japan),
636 they were reacted with anti-tgDeV DAg, anti-mmDeV DAg, or anti-actin (Sigma
637 Aldrich) antibodies as primary antibodies, followed by reaction with horseradish
638 peroxidase (HRP)–conjugated secondary antibodies (Cell Signaling Technology;
639 Danvers, MA, USA).

640

641 *Indirect immunofluorescence assay (IFA)*

642 The cells were fixed in 4% paraformaldehyde (Wako) and then permeabilized using 0.3%
643 Triton X-100 (MP Biomedicals; Santa Ana, CA, USA). After blocking the cells by
644 incubation in PBS containing 1% bovine serum albumin (BSA) (KAC; Kyoto, Japan),
645 they were treated with the primary antibodies against HDAg, tgDeV DAg, or mmDeV
646 DAg and then incubated with Alexa555-conjugated secondary antibody (Thermo Fisher
647 Scientific), together with DAPI (Nacalai tesque). To detect deltavirus-positive cells, the
648 fluorescence signal was observed using fluorescence microscopy, BZ-X710 (KEYENCE;
649 Osaka, Japan). High magnification examination of the subcellular localization was
650 performed using confocal microscopy, LSM900 (ZEISS; Oberkochen, Germany).

651

652 *Deltavirus preparation and infection assay*

653 HDV was produced from the culture supernatants of Huh7 cells transfected with HDV
654 (pSVLD3) and HBs (pT7HB2.7) expressing plasmid, as described previously (32, 66).
655 tgDeV and mmDeV were also subjected to the same assay. The supernatants of
656 transfected cells were collected at 6, 9, and 12 days post-transfection, and they were then
657 filtrated and concentrated using 0.45- μ m filters and Amicon Ultra (Merck Millipore),
658 according to the manufacturer's instructions. The concentrated supernatants were
659 inoculated into HepG2-NTCP cells with 5% PEG8000 (Sigma Aldrich) for 24 h followed
660 by washing to remove free viruses. The inoculated cells were cultured for 6 days and used
661 for the downstream analyses.

662

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670 for woodchuck were downloaded from silhouetteAC (<http://www.silhouette-ac.com/>).

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679

680 **Author contributions**

681 MH conceived the study. MI, KW, and MH designed the study. MI conducted cell culture

682 experiments. MH, JK, SK performed *in silico* analyses. YS, YTL, HLW, KW, and MH

683 prepared and analyzed animal specimens. All the authors analyzed and discussed the data.

684 MI and MH wrote the manuscript.

685

686 **Figure legends**

687 **Figure 1. Genome organization of novel deltaviruses.**

688 Genomes of **(a)** tgDeV, mmDeV, and ovDeV (complete genomes) and **(b)** scDeV and
689 egDeV (partial genomes). Annotations (ORF, poly-A signal, and ribozymes) are shown
690 by colored arrow pentagons. The numbers indicate nucleotide positions. **(c)**
691 Self-complementarities of novel deltaviruses. The predicted RNA structures were
692 visualized using the Mfold web server (58). Red, blue, and green arcs indicate G-C, A-U,
693 and G-U pairs, respectively.

694

695 **Figure 2. Amino acid sequence characterization of putative delta antigens of novel** 696 **deltaviruses.**

697 **(a)** Alignment and functional features of the putative S-HDAg and DAgs of
698 representative HDVs and novel deltaviruses. (Putative) functional domains are shown by
699 colored boxes. Me: arginine methylation site, Ac: lysine acetylation site, P: Serine
700 phosphorylation site. **(b)** ovDeV mRNA (upper panel) and a possible A-to-I RNA-editing
701 site (lower panel). Consensus ovDeV-DAg mRNA sequence and mapped read sequence
702 with potential RNA-edited nucleotides (blue boxes). Pink boxes indicate the ORF of
703 ovDeV DAg. **(c)** Deduced amino acid sequences of ovDeV-DAg proteins translated from
704 the viral mRNA with or without RNA-editing. The blue letter shows the possible
705 RNA-editing site.

706

707 **Figure 3. Mapping coverages of original short reads of each contig.**

708 Mapped read graphs of **(a)** tgDeV, **(b)** mmDeV, and **(c)** ovDeV. Lines, arrow pentagons,
709 and arrowheads indicate viral genomes, ribozymes, and poly(A) signals, respectively.

710 The numbers above the graphs show nucleotide positions. The light pink box indicates a
711 low read depth region in the putative transcript of tgDeV.

712

713 **Figure 4. Interfamily transmission of deltaviruses among passerine birds.**

714 **(a–c)** RT-PCR detection of a deltavirus from *Lonchura striata*. **(a)** Plasmid used for the
715 establishment of real-time PCR detection system for tgDeV and **(b)** the tgDeV circular
716 genome. The blue arrows indicate the primers used for endo-point RT-PCR detection. **(c)**
717 Endo-point RT-PCR for detection of the circular deltavirus genome. M, 100-bp ladder
718 marker. **(d)** Pairwise nucleotide identities between deltaviruses detected in passerine
719 birds. **(e)** Phylogenetic tree of passerine birds positive for deltaviruses. Phylogenetic tree
720 of birds and deltavirus infections are indicated. MYA: million years ago.

721

722 **Figure 5. Phylogenetic analysis of deltaviruses.**

723 **(a)** Heat map of pairwise amino acid sequence identities between deltaviruses. **(b)** The
724 phylogenetic tree was inferred by the maximum likelihood method using an amino acid
725 sequence alignment of representative deltaviruses. Known phenotypes (RNA-editing and
726 expression of the large isoform of DAg protein) and helper virus(es) of each virus are
727 shown on the right. Note that the SDeV phenotypes are shown in gray letters, because
728 there is insufficient information, evidence, or both for the RNA-editing and L-DAg
729 expression. The deltaviruses identified in this study are indicated by the blue circles.
730 Bootstrap values >70 are shown. SDeV: snake deltavirus, RDeV: rodent deltavirus.

731

732 **Figure 6. Detection of tgDeV DAg and mmDeV DAg in cells ectopically expressing**
733 **the tgDeV or mmDeV dimer genome.**

734 **(a and b)** Western blotting analysis of Huh7 or WCH-17 cells transfected with a tgDeV or
 735 mmDeV dimer-sequence expression plasmid. The numbers on the left side of panels
 736 indicate the size marker of protein (kDa). **(c–f)** Indirect immunofluorescence analysis of
 737 the expression of tgDeV or mmDeV DAg protein. The cells were observed using
 738 fluorescent microscopy **(c and d)** or a confocal microscopy **(e and f)**. Blue; DAPI, Red;
 739 tgDeV or mmDeV DAg. Scale bars = 50 μ m (c and d) and 5 μ m (e and f).

740

741 **Figure 7. No infectious particle of tgDeV and mmDeV was produced by**
 742 **supplementation of HBV envelop proteins.**

743 **(a)** Quantification of deltavirus RNAs in culture supernatants. HDV, tgDeV, or mmDeV
 744 expression plasmid was transfected with or without plasmid expressing HBV envelope
 745 proteins into Huh7 cells. Viral RNA levels in supernatants were quantified using
 746 quantitative RT-PCR (n = 3). **(b and c)** HepG2-NTCP cells were incubated with the
 747 culture supernatants of the transfectants for 24 h in the presence or absence of 500 nM
 748 Myrcludex B (MyrB), an inhibitor of HBV envelope-dependent viral entry. The cells
 749 were cultured for an additional 6 days, and viral RNA levels and protein expression were
 750 analyzed using quantitative RT-PCR (n = 3) **(b)** and IFA **(c)**, respectively. The numbers in
 751 **(c)** correspond to those of **(b)**. Blue, DAPI; Red, HDV; tgDeV, or mmDeV DAg. Scale
 752 bar = 50 μ m.

753

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Table 1. Summary of novel deltaviruses

Virus name		Host species	Tissue	SRA	DDBJ	Contig length (nt)	GC content (%)	BLASTx best hit		
				accession	accession			Virus name	Accession	Identity (%)
Taeniopygia guttata DeV	tgDeV	<i>Taeniopygia guttata</i>	Scapulohumeralis caudalis	SRR2545946	BR001665	1706	56.6	Rodent deltavirus	QJD13558	63.3
Marmota monax DeV	mmDeV	<i>Marmota monax</i>	Liver	SRR2136906	BR001661	1712	53.4	Hepatitis delta virus	AIR77039	60.0
Odocoileus virginianus DeV	ovDeV	<i>Odocoileus virginianus</i>	Pedicle	SRR4256033	BR001662	1690	56.4	Hepatitis delta virus	AHB60712	66.7
Erythrura gouldiae DeV	egDeV	<i>Erythrura gouldiae</i>	Skin	SRR7504989	BR001660	596	59.4 ^{a)}	Rodent deltavirus	QJD13555	63.5
Serinus canaria-associated DeV	scDeV	<i>Serinus canaria</i>	Skin	SRR2915371	BR001664	761	54.4 ^{a)}	Hepatitis delta virus	AIR77012	36.0
Pardaliparus venustulus DeV	pvDeV	<i>Pardaliparus venustulus</i>	Lung, Kidney, Cardiac muscle, Flight muscle, Liver	SRR7244693 SRR7244695 SRR7244696 SRR7244697 SRR7244698	BR001663	1708	55.8	Rodent deltavirus	QJD13562	62.4
Lonchura striata DeV	lsDeV	<i>Lonchura striata</i> var. <i>domestica</i>	Blood	-	LC575944	1708	56.2	Rodent deltavirus	QJD13555	62.9

a) GC content of the partial genome sequences.

Table 2. Detection of deltavirus-derived reads in RNA-seq data.

			Host		RPM ^{a)} (read per million)	Tissue
Virus	BioProject/ BioStudy	SRA	Taxonomy			
			Family	Species		
tgDeV	PRJNA297576	SRR2545943	Estrildidae	<i>Taeniopygia guttata</i>	10.28	Pectoralis
		SRR2545944			1.02	Scapulohumeralis caudalis
		SRR2545946			56.73	Scapulohumeralis caudalis
	PRJNA558524	SRR9899549	Emberizidae ^{b)}	<i>Emberiza melanocephala</i>	3.11	Blood
	PRJNA470787	SRR7244693	Paridae	<i>Pardaliparus venustulus</i>	10.68	Lung
		SRR7244695			2.07	Kidney
		SRR7244696			2.65	Cardiac muscle
		SRR7244697			7.12	Flight muscle
		SRR7244698			1.77	Liver
	PRJNA478907	SRR7504989	Estrildidae	<i>Erythrura gouldiae</i>	1.07	Skin
mmDeV	PRJNA291589	SRR2136906		<i>Marmota monax</i>	70.86	Liver
		SRR2136907			63.08	Liver
		SRR2136916			1.02	Liver
	SRP011132	SRR437934			46.34	PBMC
		SRR437938			19.83	PBMC
ovDeV	PRJNA317745	SRR4256033		<i>Odocoileus virginianus</i>	180.73	Pedicle
scDeV	PRJNA300534	SRR2915371		<i>Serinus canaria</i>	9.79	Skin

The full version of the table is available as Supplementary Table 3.

a) This table only shows the samples with RPM >1.

b) Emberizidae is regarded as the subfamily Emberizinae of the family Fringillidae in TimeTree.

Table 3. Coexisting viruses in deltavirus-positive SRAs

SRA accession	Host		Virus name	Envelope	Deltavirus infection
	Species	Common name			
SRR2545944	<i>Taeniopygia guttata</i>	Zebra finch	Serinus canaria polyomavirus	-	tgDeV
SRR5001849	<i>Taeniopygia guttata</i>	Zebra finch	Serinus canaria polyomavirus	-	tgDeV
SRR5001850	<i>Taeniopygia guttata</i>	Zebra finch	Serinus canaria polyomavirus	-	tgDeV
SRR5001851	<i>Taeniopygia guttata</i>	Zebra finch	Serinus canaria polyomavirus	-	tgDeV
SRR2915371	<i>Serinus canaria</i>	Common canary	Canary bornavirus 3	+	scDeV
			Canary circovirus	-	
SRR7504989	<i>Erythrura gouldiae</i>	Gouldian finch	Erythrura gouldiae polyomavirus 1	-	egDeV

a

b

c

a

b

c

a

b

0.05

Fig. 6

a

b

c

Blue: nucleus Red: DAg