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BRIEF REPORT

A gyrovirus infecting a sea bird

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Abstract We characterized the genome of a highly divergent gyrovirus (GyV8) in the spleen and uropygial gland tissues of a diseased northern fulmar (*Fulmarus glacialis*), a pelagic bird beached in San Francisco, California. No other exogenous viral sequences could be identified using viral metagenomics. The small circular DNA genome shared no significant nucleotide sequence identity, and only 38-42 % amino acid sequence identity in VP1, with any of the previously identified gyroviruses. GyV8 is the first member of the third major phylogenetic clade of this viral genus and the first gyrovirus detected in an avian species other than chicken.

Gyroviruses are small, non-enveloped DNA viruses with a single-stranded circular genome of ~2 kb, currently classified in the genus *Gyrovirus* within the family *Circoviridae*. Since gyroviruses have a negative-sense circular genome and a genome organization resembling that of members of the family *Anelloviridae*, the reassignment of the genus *Gyrovirus* to the family *Anelloviridae* has been proposed [10]. The first gyrovirus genome, that of the prototype chicken anemia virus (CAV), was described in 1979 [21]. CAV is a widespread pathogen of chickens that causes clinical disease and subclinical immunosuppression affecting CD8+ T lymphocytes, leading to significant losses in the poultry industry [1, 27, 29]. Since 2011, using viral metagenomics or rolling-circle amplification, several new gyrovirus genomes have been identified in chicken sera and tissue, human feces and skin swabs, and in animal feces, including human gyrovirus 1 (HGyV1), the closely related avian gyrovirus 2 (AGV2), and GyV3 through 7 [4, 7–9, 24, 26, 28, 36, 37] (Table 1). Many of these gyroviruses have been reported in chickens (CAV, HGyV1/AGV2, GyV3, 4, and 7) [4, 36]. CAV shows high resistance to inactivation [34], and DNA from CAV, HGyV1/AGV2, GyV3 and 4 has also been reported in feces of humans and other mammals, indicating possible dietary sources from consuming chicken [4, 7, 8, 22, 24, 31, 37]. Testing for HGyV1/AGV2 DNA in human blood samples has yielded conflicting results [2, 17, 18, 22], possibly complicated by its detection on human skin [28]. Whether HGyV1/AGV2 DNA in human plasma reflects actual viremia, skin contaminated with gyrovirus introduced into blood samples during phlebotomy, or transit of virus from infected food (chicken) in the gut is currently unknown. There are currently no reports of human antibodies to gyroviruses.

In this study, we used sequence-independent amplification and deep sequencing to investigate the potential viral etiology of disease in a northern fulmar (*Fulmarus glacialis*) stranded on Ocean Beach, San Francisco, California, with head tilt and ataxia. It was euthanized 7 days later on Jan 20, 2014, due to persistent ataxia and lack of improvement, and the carcass was submitted to the Anatomic Pathology Service at the School of Veterinary

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Table 1 Summary of gyrovirus sequences

Gyrovirus	Abbr.	Source	Genome characterization method	Genome (bp)	VP1 (aa)	VP2 (aa)	VP3 (aa)	Accession no.	Reference
Chicken anemia virus	CAV	Virus culture	Cloning & Sanger sequencing	2319	449	216	121	NC_001427	Noteborn, et al., 1991 [21]
Avian gyrovirus 2	AGV2	Chicken serum	RCA, cloning & Sanger sequencing	2383	460	231	124	NC_015396	Rijsewijk, et al., 2011 [26]
Human gyrovirus 1	HGyV1	Human skin swab	Metagenomics	2315	465	231	124	NC_015630	Sauvage, et al., 2011 [28]
Gyrovirus 3	GyV3	Human feces	Metagenomics	2359	463	239	125	NC_017091	Phan, et al., 2012 [24]
Gyrovirus 4	GyV4	Human feces & chicken meat	Metagenomics	2034	352	217	85[1]	NC_018401	Chu, et al., 2012 [4]
Gyrovirus Tu243	GyV5	Human feces	Metagenomics	2020	356	231	89[1]	NC_022788	Phan, et al., 2013 [24]
Gyrovirus Tu789	GyV6	Human feces	Metagenomics	2282[2]	453	225	111	NC_022789	Phan, et al., 2013 [24]
Gyrovirus GyV7-SF	GyV7	Chicken meat	Metagenomics	2439	465	238	130	NC_025215	Zhang, et al., 2014 [36]
Gyrovirus NoFu	GyV8	Northern Fulmar tissues	Metagenomics	2218[2]	478	232	103[1]	KR137527	Li, et al., 2015 [15]

[1] VP3 protein with no apoptin domain

[2] Nearly complete genome sequence with part of the non-translated region missing due to high GC content

Medicine, University of California, Davis, for routine necropsy and diagnostic tests. The bird was in thin body condition and was found by histopathologic analysis to have acute ulceration and cellulitis of the foot webbing, microhemorrhages of serosal surfaces, corneal ulceration, brain microhemorrhage, and bursal depletion. Fresh, frozen spleen and uropygial gland tissue were processed as described previously [13, 14]. Briefly, the tissue samples (~25 mg) were immersed in 1 ml of ice-cold Hank's balanced saline solution and disrupted with a tissue homogenizer for 30 seconds on ice. The resulting homogenates were placed on dry ice for 5 minutes and thawed at room temperature. Freezing and thawing were then repeated twice. The tissue homogenates were then centrifuged at 12,000*g* for 5 minutes and the supernatants were filtered through a 0.45-μm filter (Millipore) to remove host cellular debris. The viral particles containing filtrates were digested with a mixture of DNases and RNases to reduce the concentration of unprotected nucleic acids. Viral nucleic acids, protected within viral capsids, were then extracted using a MagMAX Viral RNA Isolation Kit (Ambion) [15]. Extracted viral nucleic acids were protected from RNAse degradation by addition of 40 U of RNase inhibitor (Fermentas) and stored at −80 °C. Extracts from the spleen and uropygial gland were pooled and a nucleic acid library was constructed using a Nextera XT DNA sample preparation kit (Illumina) and then sequenced using the MiSeq Illumina platform (paired-end 2 × 250 bp).

Sequence reads were debarcoded using vendor software from Illumina. A total of ~294,000 reads were generated. A virus discovery pipeline running on a 32-node Linux cluster was used to process the data. Bacterial reads were subtracted by mapping the reads to bacterial RefSeq genomes release 66 using Bowtie 2 [12]. Clonal reads were removed, and low-sequencing-quality tails were trimmed, using a Phred quality score of 10 as the threshold. Adaptors were trimmed using the default parameters of VecScreen [19]. The cleaned reads were assembled *de novo* using multiple sequence assembly programs [11, 16, 20, 30]. The assembled contigs and singlets were translated and compared to a viral proteome database (consisting of all annotated complete or nearly complete viral genome sequences) using BLASTx. The significant hits to viruses were then aligned to a non-virus, non-redundant (NVNR) universal proteome database using BLASTx. Hits with E-values showing a better match with NVNR than with viruses were removed [6].

Two reads forming one contig (~350 nt) detected in the tissue pool from the diseased bird had significant similarity to avian gyrovirus 2 (BLASTx E-value < 1e-10). Other than these gyrovirus hits, no significant matches were found to other viruses except for retroviruses. These hits

were attributed to the reverse transcriptase reagent and to avian germ line endogenous retroviral sequences. The presence of the novel gyrovirus was confirmed by PCR, using re-extracted DNA from both the spleen and the uropygial gland. The rest of the viral genome was then amplified using inverse nested PCR, and the amplicon was sequenced by the Sanger method. Due to the high GC content, the non-translated region (NTR) could not be sequenced despite multiple attempts with different GC-optimized buffers and sequencing from a plasmid subclone. The assembled, nearly complete genome sequence was referred to as northern fulmar gyrovirus, which was tentatively named gyrovirus 8 (GyV8, GenBank accession no. KR137527). The putative ORFs of the GyV8 were predicted using Geneious 7 (Biomatters).

The nearly complete genome sequence of GyV8 was 2218 nt long, missing an estimated ~100 nucleotides from the GC-rich region. The genome organization showed typical features of gyroviruses (Fig. 1A), with three major overlapping ORFs (>300 nt) in the same orientation. Two ORFs were predicted to encode the viral capsid protein VP1 (478 aa) and non-structural protein VP2 (232 aa), which were similar in size to the VP1 and VP2 protein of previously identified gyroviruses, ranging from 352 to 465 aa and 216 to 239 aa, respectively (Table 1). The third ORF encoded the putative VP3 protein, which was 103 amino acids long and showed no significant amino acid sequence identity to VP3 of other gyrovirus or other proteins in GenBank non-redundant database (Fig. 1A). The VP3 apoptin protein from CAV and AGV2/HGyV1 induces apoptosis in human cancer cells [3, 5, 33, 35]. The incomplete NTR (minus the GC region) was 335 nt long, and a potential TATA box (CTATATAAG) was identified using a promoter prediction program [25].

Sequence alignments of the VP1 proteins of GyV8 and previously described gyroviruses showed that VP1 of GyV8 shared 38-42 % amino acid sequence identity. Several highly conserved amino acid motifs were identified in VP1, including VRLPNPYN, SKXGGP, WWRWXL and GGWXLFRH, FXPVASLL. The conserved motif

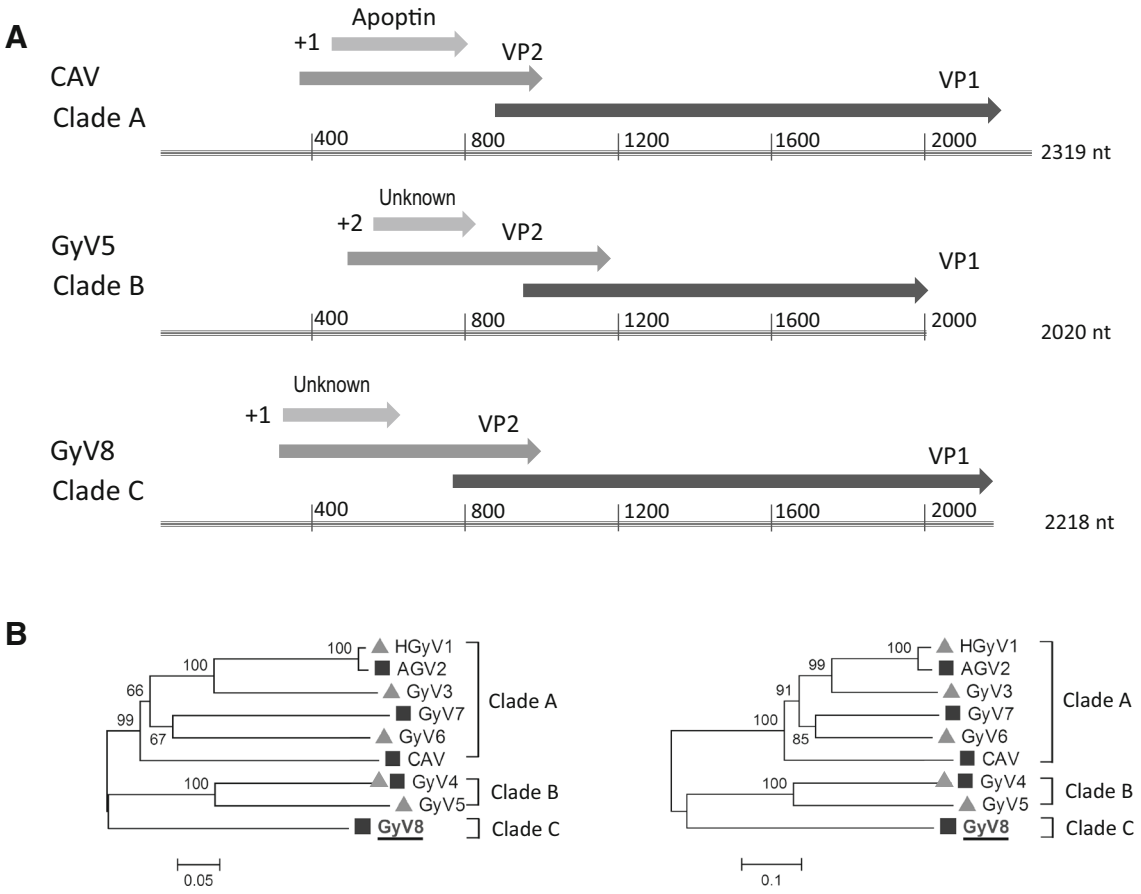

Fig. 1 Genome organization and phylogenetic analysis of gyroviruses in different clades. **A**) Genome organization of chicken anemia virus, gyrovirus 5, and northern fulmar gyrovirus. **B**) Phylogenetic trees generated with VP1 and VP2 protein sequences of representative gyroviruses (GenBank accession numbers are shown in Table 1). The scale indicates amino acid substitutions per position. Bootstrap values >60 % are shown. The square and triangle indicate that the virus was initially discovered in blood/tissue and feces/skin swab, respectively

TLX_2AQ for rolling-circle replication was located near the VP1 C-terminus but was modified as VLX_2AQ. The VP2 of GyV8 showed only 9-15 % amino acid sequence identity to those of known gyroviruses. The putative phosphatase motif CX_5R [23] of VP2 was absent, but the $WX_7HX_3CXCX_5H$ motif, which was conserved in previously identified gyroviruses and anelloviruses, was identified [32].

Phylogenetic analysis based on complete VP1 and VP2 amino acid sequence alignments of GyV8 and other known gyroviruses were performed by the neighbor-joining method in MEGA 5, using amino acid p-distances with 1,000 bootstrap replicates (Fig. 1B). Both the VP1 and VP2 trees showed that gyroviruses clustered into three major clades. CAV along with AGV2/HGyV1, GyV3, 6 and 7 formed clade A, with larger genomes (2315 - 2383 nt) and the apoptin-encoding VP3 ORF in +1 frame relative to underlying VP2 ORF. GyV4 and 5 formed clade B [9], with smaller genomes (2020 - 2034 nt) and a VP3 ORF in +2 frame relative to underlying VP2 ORF with no sequence similarity to apoptin. GyV8 formed clade C, with a VP3 ORF in +1 frame relative to the underlying VP2 ORF. GyV8 VP3 showed no sequence similarity to either apoptin of clade A or the VP3 of unknown function of clade B gyroviruses (GyV4,5).

The gyrovirus KM348009, recently described in ferret feces, shared 76 % genome-wide nucleotide sequence identity with GyV3, and both viruses were considered different variants/genotypes of the same species [7, 8, 24]. A species cutoff value of <75 % genome-wide nucleotide sequence identity was proposed [8]. Using BLASTn, the genome of GyV8 only showed detectable nucleotide similarity to those of other gyroviruses over short regions totaling ~11 % of its genome, readily qualifying GyV8 as the prototype of a new gyrovirus species. GyV8 is also the first gyrovirus reported to infect an avian species other than chicken (*Gallus gallus domesticus*).

In an effort to correlate GyV8 with the lesions in the infected sea bird, 12 other fulmars were necropsied. These birds, stranded between Monterey, CA, and Dillon Beach, CA, from October 2013 to January 2014, either died or were euthanized during rehabilitation. Six of the birds examined and collected had clinical or histologic lesions that were either similar or overlapping with those of the sentinel bird. Both spleen and uropygial glands in these birds were used as templates for specific PCR amplification of GyV8. No GyV8 DNA was amplified. Whether or not GyV8 contributed to the morbidity of the sentinel bird is therefore speculative, and further studies are needed to determine the host range and disease association of GyV8.

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