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Novel Gammaherpesviruses in North American Domestic Cats, Bobcats, and Pumas: Identification, Prevalence, and Risk Factors

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ABSTRACT

Gammaherpesviruses (GHVs) are a diverse and rapidly expanding group of viruses associated with a variety of disease conditions in humans and animals. To identify felid GHVs, we screened domestic cat (*Felis catus*), bobcat (*Lynx rufus*), and puma (*Puma concolor*) blood cell DNA samples from California, Colorado, and Florida using a degenerate pan-GHV PCR. Additional pan-GHV and long-distance PCRs were used to sequence a contiguous 3.4-kb region of each putative virus species, including partial glycoprotein B and DNA polymerase genes. We identified three novel GHVs, each present predominantly in one felid species: *Felis catus* GHV 1 (FcaGHV1) in domestic cats, *Lynx rufus* GHV 1 (LruGHV1) in bobcats, and *Puma concolor* GHV 1 (PcoGHV1) in pumas. To estimate infection prevalence, we developed real-time quantitative PCR assays for each virus and screened additional DNA samples from all three species ($n = 282$). FcaGHV1 was detected in 16% of domestic cats across all study sites. LruGHV1 was detected in 47% of bobcats and 13% of pumas across all study sites, suggesting relatively common interspecific transmission. PcoGHV1 was detected in 6% of pumas, all from a specific region of Southern California. The risk of infection for each host varied with geographic location. Age was a positive risk factor for bobcat LruGHV1 infection, and age and being male were risk factors for domestic cat FcaGHV1 infection. Further characterization of these viruses may have significant health implications for domestic cats and may aid studies of free-ranging felid ecology.

IMPORTANCE

Gammaherpesviruses (GHVs) establish lifelong infection in many animal species and can cause cancer and other diseases in humans and animals. In this study, we identified the DNA sequences of three GHVs present in the blood of domestic cats (*Felis catus*), bobcats (*Lynx rufus*), and pumas (*Puma concolor*; also known as mountain lions, cougars, and panthers). We found that these viruses were closely related to, but distinct from, other known GHVs of animals and represent the first GHVs identified to be native to these feline species. We developed techniques to rapidly and specifically detect the DNA of these viruses in feline blood and found that the domestic cat and bobcat viruses were widespread across the United States. In contrast, puma virus was found only in a specific region of Southern California. Surprisingly, the bobcat virus was also detected in some pumas, suggesting relatively common virus transmission between these species. Adult domestic cats and bobcats were at greater risk for infection than juveniles. Male domestic cats were at greater risk for infection than females. This study identifies three new viruses that are widespread in three feline species, indicates risk factors for infection that may relate to the route of infection, and demonstrates cross-species transmission between bobcats and pumas. These newly identified viruses may have important effects on feline health and ecology.

Viruses of the *Herpesviridae* family are double-stranded DNA viruses with large (>100-kb) viral genomes and are classified into three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae* (1). Gammaherpesviruses (GHVs) are a rapidly expanding group of viruses that exist in a wide range of animal hosts (2). While they are a diverse group of viruses, several common features exist. GHVs typically establish latent infection that persists for the life of the infected host, during which little gene expression or virus production occurs (3). However, viral gene expression can be reactivated, resulting in production of progeny virus (4). While GHVs may infect many cell types, they often are lymphotropic and under certain conditions can induce lymphoproliferative disorders, including lymphoma, as well as nonlymphoid cancer (5, 6). Most GHVs studied to date do not cause

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overt disease under normal conditions in their adapted host but, rather, are associated with disease under altered conditions, such as immunosuppression or transmission to a nonadapted species. For instance, the two GHVs of humans, Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV), are often asymptomatic under normal conditions but cause cancer and lymphoproliferative disorders in immunosuppressed individuals, such as those with HIV/AIDS (7–9). Likewise, malignant catarrhal fever of cattle and other ruminants occurs when alcelaphine herpesvirus 1 and ovine herpesvirus 2 infect species other than their native host species (wildebeest and sheep, respectively) and cause fatal lymphoproliferative disease (6, 10). Thus, GHVs can impact human and animal health. The presence of GHVs and their pathogenesis remain unexplored for many mammalian species, including felids.

The family *Felidae* includes more than 40 species located throughout the world (11). Ehlers and colleagues identified a GHV DNA sequence in an African lion (*Panthera leo*) (2), and several studies have reported that domestic cats can be infected by bovine herpesvirus 4 (BoHV4) (12–14). Antibodies to EBV have been detected in domestic cats (*Felis catus*) (15) (J. Beatty, unpublished data), suggesting that an EBV-like virus could be present in cats. Furthermore, cats infected with feline immunodeficiency virus (FIV) are at increased risk for B-cell lymphomas that resemble HIV-associated lymphoma (16, 17). HIV-associated lymphomas are typically caused by EBV or KSHV (7), suggesting the possibility of an unidentified GHV participating in FIV-associated lymphoma. These reports suggest the presence of GHVs in a limited number of felids. However, despite attempts to do so (18, 62), no native GHVs have been identified in the majority of felid species, including the domestic cat, which is a major companion animal worldwide.

In this study, we utilized a degenerate pan-GHV PCR strategy to search for GHVs in felids present in North America, including the domestic cat, bobcat (*Lynx rufus*), and puma (*Puma concolor*; also known as mountain lion, cougar, and panther). We identified the DNA sequences of three previously uncharacterized GHVs, each present predominantly in one particular cat species. Using additional degenerate and virus-specific PCRs, we expanded the DNA sequence of each virus to include 3.4 kb of the glycoprotein B (gB) and DNA polymerase (DNApol) genes and compared these sequences to those of known GHVs by phylogeny. To study the prevalence of these viruses, we developed real-time quantitative PCR (qPCR) assays for the detection of each virus and screened 282 blood cell DNA samples from the three cat species collected in California, Colorado, and Florida. These analyses demonstrated the variable prevalence and species specificity of the three viruses as well as significant correlations with geographic location and cat age and sex.

MATERIALS AND METHODS

Ethics statement. Animal procedures were approved by the Colorado State University Animal Care and Use Committee or appropriate institutional, local, and state agencies, prior to initiation of sample collection as previously described (19).

Sample collection and DNA preparation. Samples were derived from six sites spanning three states (California, Colorado, and Florida). Ventura County (VC; also incorporating some of Los Angeles County) and Orange County (OC) sites represent landscapes surrounding the Santa Monica and Santa Ana Mountains north and south of Los Angeles, California, respectively. Samples were also collected from the eastern portion of San Diego and Riverside Counties (SDRC), California. Colorado Rocky Mountain sites included the Western Slope (WS), located around Montrose and Grand Junction, and the Front Range (FR; domestic cat samples only), including Boulder and the northwest Denver metropolitan area. Samples from southern Florida were collected from areas around Fort Myers and Naples, including Okaloacoochee Slough State Forest, the Florida Panther National Wildlife Refuge, Big Cypress National Preserve, Picayune Strand State Forest, and Fakahatchee Strand Preserve State Park. Climatic and natural vegetation communities vary among all three states. California sites represent Mediterranean climatic conditions with coastal California sage scrub, chaparral, riparian and coastal oak woodlands, and annual grasslands. Colorado sites are cooler than California sites and semiarid with vegetation characterized by coniferous woodlands and forests primarily interspersed with aspens. Southern Florida is humid subtropical or tropical savannah with vegetation communities consisting of pine flatwoods, south Florida rockland, cypress domes and strands, dwarf cypress, prairies, mixed hardwood swamps, hardwood hammocks, freshwater swamps, and mangroves.

Blood samples from sympatric populations of wild and domestic felids were collected as part of other ongoing studies and shipped to the Feline Retrovirology Research Laboratory at Colorado State University (19). All samples entered into the study were derived from free-ranging healthy individuals and were collected as part of ongoing ecological studies for bobcat and puma and through trap-neuter-release clinics for domestic cats on admission to shelters (19, 20). Blood samples were initially stored in EDTA tubes and were either refrigerated at 4°C or kept on ice until the return from the field, where they were temporarily frozen at −20°C and later transferred to −80°C. Total DNA was extracted from thawed whole blood or buffy coat using a DNeasy blood and tissue kit (Qiagen, Valencia, CA). Samples were collected over a 2- to 3-year period at each site, primarily between 2001 and 2012. Animal sex and location were recorded at the time of capture, and age (young, age $<$2 years; adult, age $>$2 years) was estimated on the basis of the size and weight of the animal and dental wear (21).

Several DNA samples were obtained for testing the specificity of qPCR assays. Archived blood cell DNA from specific-pathogen-free domestic cats was obtained from three previous studies (22–24). BoHV4 and feline herpesvirus 1 (FeHV1) DNAs were extracted from infected cell culture supernatants (provided by Hana Van Campen and Sandra Quackenbush, respectively, both of Colorado State University) using a QIAamp DNA blood kit (Qiagen). Archived bovine lymph node DNA containing bovine herpesvirus 6 (BoHV6) was obtained from Joel Rovnak, Colorado State University. The presence of the intended herpesviral DNA was confirmed by PCR (R. Troyer and J. Beatty, unpublished data).

PCR amplification and sequencing. Degenerate nested pan-GHV PCR to amplify a portion of the gB gene was conducted with previously described primer set RH-gB (2). In the first round, 5 μl DNA (50 to 500 ng total) was added to 50-μl reaction mixtures containing 2 units Platinum *Taq* polymerase (Life Technologies, Carlsbad, CA), 1 μM primers 2759s and 2762as, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, and 1× PCR buffer (Life Technologies). Cycling conditions were as follows: initial denaturing at 94°C for 2 min, followed by 45 cycles of 94°C for 30 s, 46°C for 30 s, and 72°C for 30 s and then a 7-min extension at 72°C. In the second round, 2 μl of the first-round reaction product was used as the template and reactions were conducted under conditions identical to those described above using primers 2760s and 2761as. Stringent measures were taken to avoid cross-contamination, including use of separate areas for reaction mix preparation and template addition. Negative-control reactions (water template) were consistently negative. PCR products of ~500 bp were purified with a QIAquick PCR purification kit (Qiagen) and sequenced in both directions by the Colorado State University Proteomics Facility. After removal of the primer sequence, 453-bp unique gB sequences were compared to other GHV gB sequences using the BioEdit (25) and NCBI BLAST programs.

Each virus required a different protocol for the amplification of 160 to

TABLE 1 Oligonucleotides used in this study

Primer set	Name	Use[a]	Sequence
Perca-DNApol	LSSG	1st for	TCACCTCATGATTACCAAACATTTCATTTGAGTAGTGG
	GDTD2	1st and 2nd rev	TGACATTCTATAAAMASAGAGTCIGTRTCICCRTA
	VYGA2	2nd for	ACATGTAACGCAGTITAYGGITTYACIGG
Puma-DNApol	VVDFAS	1st for	CAATAACCCCGTTCTTGTIGTIGAYTTYGCIAG
	VYGFTG	1st rev	TGATGCCACICCIGTRAAICCRTAIAC
	QAHNLC	2nd for	CCAAGTATAATACARGCICAYAAYCTITGYTA
	KQQLAI	2nd rev	TGAATTACATGTTACTTTIATIGCIAGYTGYTGYTT
Puma-3′end	PGHV-F11	1st for	ATGATTCCCTGGCACGTTAC
	GDTD1B	1st and 2nd rev	CGGCATGCGACAAACACGGAGTCNGTRTCNCCRTA
	PGHV-F12	2nd for	CTATCCAGCCTCCTCACAGC
FcaGHV1 long	FGHV-F1	1st for	ACCTGCACCAGAGCATGAGA
	FGHV-R4	1st rev	ATCACCCTGAAACTGGCGTTA
	FGHV-F2	2nd for	TACTCCAGACCCATCGTCACAT
	FGHV-R5	2nd rev	CGCCTCCCTTCGTAGGTTATAGTT
LruGHV1 long	LGHV-F15	1st for	GGGGATGTGATTTCGGTGAC
	LGHV-R13	1st rev	TCACCCTGAAACTGGCGTTA
	LGHV-F16	2nd for	GCATGAGAGTTCCAGGTCCA
	LGHV-R14	2nd rev	GCACAGTTGAGTGGCCTTTG
PcoGHV1 long	PGHV-F9	1st for	CAAGACTATGAAAACCAAGGAACC
	PGHV-R11	1st rev	GCTGTGAGGAGGCTGGATAG
	PGHV-F10	2nd for	TTGTCCAACACACATATCGAAG
	PGHV-R12	2nd rev	TGTACCTCCACTTATGTTAAATGATTC
FcaGHV1 qPCR	FGHV-F3	For	ACATCTTCACTGGACAACTGG
	FGHV-R3	Rev	GTGCATTTGATGTCCTGACTG
	FGHV-P3	Probe	TGAACAGCTGAGTCTCTACAAGTCTCCA
LruGHV1 qPCR	LGHV-F3	For	CTGGACAATTGGGTCCTAGAAA
	LGHV-R3	Rev	CATTTGATGCCCTGACTGAAAG
	LGHV-P3	Probe	AGTGGAGACTTGTAGAGACTCTGCTGT
PcoGHV1 qPCR	PGHV-F9	For	CAAGACTATGAAAACCAAGGAACC
	PGHV-R9	Rev	CCGAGCTGACCCACAAATAT
	PGHV-P9	Probe	AGGCCCGCAGTAACCTTCAAGT

[a] Round of PCR (1st or 2nd) and forward primer (for), reverse primer (rev), or qPCR probe are indicated.

180 bp of the DNApol gene. The DNApol gene of *Lynx rufus* GHV 1 (LruGHV1) was amplified using previously published heminested primers: DFASA and GDTD1B in the first round and VYGA and GDTD1B in the second round (26). The reaction mix was identical to that for gB, and cycling conditions were as follows: initial denaturing at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min and then a 7-min extension at 72°C. The DNApol of *Felis catus* GHV 1 (FcaGHV1) was amplified using the Perca-DNApol primer set designed to be more percavirus specific (Table 1). PCR conditions were identical to those used to amplify LruGHV1 DNApol, except that 45 cycles and an annealing temperature of 55°C were used. The DNApol of *Puma concolor* GHV 1 (PcoGHV1) was amplified with the Puma-DNApol primer set (Table 1) using PCR conditions identical to those used to amplify LruGHV1 DNApol, except that 45 cycles, an annealing temperature of 57°C for the first round, and an annealing temperature 53°C for the second round were used. Because this PCR product was upstream of the DNApol region targeted for FcaGHV1 and LruGHV1, another PCR was required to fill in this 3′ gap for PcoGHV1 (Fig. 1). We used the Puma-3′end primer set (Table 1) with PCR conditions identical to those for the LruGHV1 DNApol PCR, except that 40 cycles and an annealing temperature of 55°C were used.

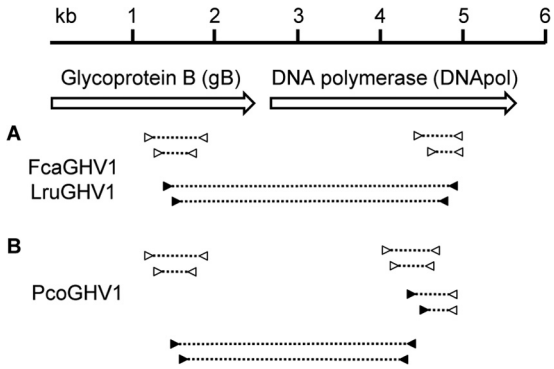

FIG 1 PCR strategy for sequencing partial gB and DNApol genes of felid gammaherpesviruses. Nested primer sets are displayed as triangles: open triangles for degenerate primers and closed triangles for virus-specific primers. A degenerate pan-GHV PCR targeting gB was used for initial detection of all viruses. (A) A degenerate panherpesvirus PCR was used to amplify a short fragment of DNApol from FcaGHV1 and LruGHV1. (B) PcoGHV1 required two degenerate PCRs to sequence DNApol. The 170- to 453-bp sequences of DNApol and gB, respectively, were used to design virus-specific primers for long-distance PCR, resulting in an ~3.4-kb bigenic sequence for each virus.

Long-distance PCR from gB to DNApol was conducted using High Fidelity Platinum *Taq* polymerase (Life Technologies) according to the manufacturer's instructions and the virus-specific nested primers listed in Table 1 at a 400 nM final concentration. Cycling conditions for LruGHV1 and FcaGHV1 were 94°C for 2 min; 7 cycles of 94°C for 30 s, 60°C for 30 s with the temperature decreasing by 0.5°C each cycle, and 68°C for 4 min; and 33 cycles of 94°C for 30 s, 57°C for 30 s, and 68°C for 4 min, followed by 68°C for 7 min. Cycling conditions for PcoGHV1 were identical, except that the annealing temperature was 57°C for the first cycle, decreased by 0.5°C each cycle for the next 6 cycles, and 54°C for the last 33 cycles. Nucleotide sequences were obtained from the long-distance PCR products by primer walking.

Phylogenetic analyses. DNApol and gB amino acid sequences derived from partial gene sequences were aligned using T-Coffee (27) with default parameters. All positions with gaps and areas with weak support for the alignment were manually removed. The resulting DNApol and gB alignments were concatenated to form a single amino acid alignment with 983 positions for phylogenetic analysis. The alignment was input into the DataMonkey server (28) to estimate the best-fit model of amino acid substitution (29), which was the model of Le and Gascuel (LG) (30). Maximum likelihood (ML) phylogenetic analyses were conducted using the PhyML program (31) applied in Geneious Pro (version 5.6) software (Biomatters, Auckland, New Zealand) with the LG substitution model and the gamma distribution with five discrete categories. ML trees were constructed using a neighbor-joining starting tree followed by a heuristic search using the nearest-neighbor interchange algorithm. The betaherpesvirus human cytomegalovirus (HCMV; human herpesvirus 5 [HHV5]) was used as an outgroup to root the tree. Bootstrap analyses were performed with 100 iterations to evaluate the support for each node.

Quantitative real-time PCR assays. Primers and 5′ 6-carboxyfluorescein-labeled and 3′ 6-carboxytetramethylrhodamine-labeled probe sets were designed to target the gB gene of each of the three felid GHVs (Table 1). The reaction mixtures were prepared with iTaq universal probes Supermix (Bio-Rad, Hercules, CA), 400 nM primers, 200 nM probe, and 50 to 500 ng of template DNA in a total volume of 25 μl. Cycling conditions for FcaGHV1 and PcoGHV1 included an initial 95°C step for 3 min, followed by 45 cycles of 95°C for 5 s and 60°C for 30 s. Conditions for LruGHV1 were identical, except that an annealing temperature of 62°C was used. Reactions were run in a 96-well format on a CFX Connect real-time system (Bio-Rad). Plasmid standards for quantitation were prepared for each virus by cloning the portion of the gB gene amplified by the degenerate pan-GHV PCR into pCR4-TOPO using a TOPO TA cloning kit (Life Technologies). Dilutions of plasmids ranging from 10^7 to 10^2 copies per reaction mixture were prepared in a background of salmon sperm DNA equivalent to 250 ng per reaction mixture, and these standards were run with each set of PCRs. Amplification efficiency for all of the assays was consistently 95 to 105%. Replicate qPCRs were run for each sample, and samples were considered positive only if both replicates were positive with greater than 3 copies per reaction (32). For quantitation of the number of copies per million cells, the number of cell equivalents for each DNA sample was determined as described by Terwee et al. (33).

To assess possible cross-reaction to feline genomic DNA, we tested archived blood DNA from 20 specific-pathogen-free domestic cats and found that all of the assays were consistently negative. To further assess specificity, we tested each assay against its intended gB target as well as DNA from the GHVs BoHV4 and BoHV6 and DNA from FeHV1, a feline alphaherpesvirus which can be present in all of these felid species. Each qPCR assay had high specificity for the intended target with no detection of BoHV4, BoHV6, or FeHV1. Prior to testing all puma and bobcat samples with both LruGHV1 and PcoGHV1 assays, we determined that these assays did not cross-react with plasmid DNA containing the gB sequence from the other virus at 10,000 copies per reaction mixture.

Statistical analyses. To compare median viral loads between groups, we used the nonparametric Kruskal-Wallis one-way analysis of variance with Dunn's multiple-comparison posttests. We calculated the prevalence of infection by dividing the number of qPCR-positive samples by the total number of samples tested and then utilized maximum likelihood estimation to determine confidence intervals (CIs) for prevalence. We examined how the probability of GHV infection was related to host sex, age, state, and site within state utilizing a general linear model with a binomial error distribution. GHV infection was predicted by sex, age, and state as independent predictors and site nested within state. For all pairwise comparisons within each predictor, we calculated individual odds ratios using unconditional maximum likelihood estimation with small sample size adjustment. All analyses were undertaken using the packages stats4, stats, and epitools in the program R (www.r-project.org).

Nucleotide sequence accession numbers. The nucleotide sequences of the novel GHVs have been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under the following accession numbers: *Felis catus* gammaherpesvirus 1, KF840715; *Lynx rufus* gammaherpesvirus 1, KF840716; and *Puma concolor* gammaherpesvirus 1, KF840717.

TABLE 2 Gammaherpesvirus glycoprotein B sequences detected in feline DNA samples by degenerate pan-GHV PCR

Host species	No. of samples	No. of samples by location	No. of GHV-positive samples
Domestic cat (*Felis catus*)	60	34 CA, 26 CO	4 (all FcaGHV1)
Bobcat (*Lynx rufus*)	34	19 CA, 15 FL	24 (all LruGHV1)
Puma (*Puma concolor*)	42	32 CA, 10 CO	6 (4 PcoGHV1, 2 LruGHV1)

RESULTS

Detection of novel gammaherpesvirus sequences in bobcats, pumas, and domestic cats. To search for herpesvirus DNA sequences present in felids, we performed degenerate nested PCR on felid blood cell DNA samples using primers which target the glycoprotein B (gB) gene of gammaherpesviruses (GHVs) (2, 34, 35). In our initial analysis, 4 of 60 domestic cat samples were PCR positive, and 453-bp gB sequences amplified from these samples were identical (Table 2). Comparison of this sequence to sequences in GenBank by BLAST analysis demonstrated homology to GHV gB sequences from other species, with the greatest similarity (80% nucleotide identity) being to mustelid herpesvirus 1 (MusHV1) of European badgers (36). The presence of this novel GHV sequence in multiple cats provided evidence for the likely existence of a novel GHV in cats. In accordance with the convention of naming herpesviruses on the basis of the host species (37), we gave this putative virus the provisional name *Felis catus* gammaherpesvirus 1 (FcaGHV1).

Bobcat samples ($n = 34$) yielded 24 gB PCR-positive samples (Table 2). Sequences from these samples were 99 to 100% identical to each other, indicating that they comprise a single viral species. A GenBank BLAST search and comparison to the FcaGHV1 sequence demonstrated that the bobcat GHV sequence had the greatest similarity to FcaGHV1 and MusHV1 (94% and 81% nucleotide sequence identity, respectively). This viral species was given the provisional name *Lynx rufus* gammaherpesvirus 1 (LruGHV1). While the LruGHV1 sequences were nearly identical to each other, we noted that at four of the five polymorphic nucleotide sites present within these sequences, the samples from Florida bobcats had a different nucleotide than the samples from California bobcats, demonstrating the presence of two closely related LruGHV1 strains from geographically distinct populations.

Puma samples ($n = 42$) yielded 6 gB PCR-positive samples (Table 2). Two of the sequences were identical to LruGHV1 sequences from bobcats, demonstrating that LruGHV1 can also infect pumas. The other four sequences associated with puma blood DNA were identical to each other and were highly divergent from the LruGHV1 and FcaGHV1 sequences (56% and 57% nucleotide identity, respectively). These sequences had the greatest similarity (82% nucleotide identity) to *Panthera leo* gammaherpesvirus 1 (PleoGHV1), the only other GHV detected in a wild felid, the African lion (2). Thus, we gave this virus the provisional name *Puma concolor* gammaherpesvirus 1 (PcoGHV1).

Extended sequencing and phylogenetic comparison to other gammaherpesviruses. To extend the length of the sequence for each felid GHV to include portions of the adjacent gB and DNA polymerase (DNApol) genes, we selected representative samples for each GHV species and used the following approach (Fig. 1): (i) amplify short segments of DNApol with degenerate panherpesviral PCR to obtain an approximately 170-bp sequence, and (ii) use short gB and DNApol sequences to design virus-specific primers for long-distance PCR. This strategy resulted in the generation of a bigenic sequence of approximately 3.4 kb (34, 35, 38) and was used to ensure that gB and DNApol sequences were derived from the same virus genome. A common panherpesviral PCR used to target DNApol for virus discovery (26, 39) amplified LruGHV1 and bovine herpesvirus 6 GHV DNA, but not FcaGHV1 or PcoGHV1. Thus, FcaGHV1 and PcoGHV1 required modified degenerate PCRs based on closely related viruses for amplification of DNApol (Fig. 1 and Table 1). The full 3.4-kb region from LruGHV1 was sequenced from a bobcat sample (from the Ventura County, CA, study site) and a puma sample (from the Western Slope, CO, study site). The sequences of these isolates differed by only a single nucleotide, further confirming that the LruGHV1 found in pumas is the same viral species found in bobcats. We identified partial open reading frames of gB and DNApol in sequences from each of the three viral species.

We aligned the novel felid GHV gB and DNApol amino acid sequences to sequences of previously described viruses for phylogenetic analysis (Fig. 2). This analysis supported the presence of multiple lineages within the GHVs (2). FcaGHV1 and LruGHV1 clustered within a lineage corresponding to the *Percavirus* genus and were most closely related to each other and MusHV1 of European badgers (Fig. 2). This genus also includes two well-described GHVs of horses, equid herpesviruses 2 and 5. While the phylogenetic analysis was restricted to GHVs for which gB and DNApol sequences were available, several short (160- to 220-bp) GHV DNApol sequences which, along with similarity to MusHV1, have 75 to 80% nucleotide sequence identity to FcaGHV1 and LruGHV1 have been reported in this region. These include GHV sequences identified in host species within the order *Carnivora*, including Darwin's fox (40), sea otter (41), oriental small-clawed otter (GenBank accession no. FJ797657), and fisher (42). Thus, it is likely that FcaGHV1 and LruGHV1 are part of a closely related subgroup of percaviruses of carnivores. PcoGHV1 was closely related to PleoGHV1 of African lions, and these viruses clustered within a lineage corresponding to the *Rhadinovirus* genus (Fig. 2). This genus includes KSHV of humans, several primate GHVs, and several other well-described viruses, such as murine gammaherpesvirus 68 (murid herpesvirus 4) of mice and bovine herpesvirus 4 of cattle.

Prevalence of novel gammaherpesvirus sequences among U.S. felids by qPCR. While the degenerate pan-GHV PCR performed well for virus discovery, the degeneracy of the primers could result in a low sensitivity for virus detection. Therefore, we designed real-time qPCR assays for each virus utilizing primers and probes targeted specifically to the gB gene of each virus. Based on the results of the pan-GHV PCR screening, in which only the FcaGHV1 sequence was found in domestic cats (Table 2), we tested domestic cat blood DNA samples using only the FcaGHV1 assay. Since we detected the LruGHV1 sequence in samples from both bobcats and pumas, all bobcat and puma samples were subjected to the LruGHV1 and PcoGHV1 assays.

The results of the qPCR assays confirmed the presence of GHV DNA in all samples that were previously identified to be GHV positive using degenerate primers. The qPCR assays also detected GHV DNA in some samples that were negative using degenerate primers. Additionally, we expanded the number of samples for each felid species to include samples from each study site (282 total samples consisting of 135 samples from domestic cats, 64 samples from bobcats, and 83 samples from pumas). The prevalence of FcaGHV1 qPCR-positive results in domestic cats was 16% (Fig. 3A). The prevalence of LruGHV1 qPCR-positive results in bobcats was 47%, and that in pumas was 13% (Fig. 3A). New blood samples from five pumas with positive results for LruGHV1 (three from Florida and two from Colorado) were tested to verify that the results reflected actual infection versus sample contamination. Four of the five new samples were also positive, confirming the original result. We detected PcoGHV1 in 6% of pumas but did not detect it in bobcats (Fig. 3A).

Viral loads, expressed as the number of GHV DNA genomes per million blood cells, were calculated from qPCR analyses. The median viral loads were similar among the GHVs in their predominant host: FcaGHV1 in domestic cats, LruGHV1 in bobcats, and PcoGHV1 in pumas (Fig. 3B). In contrast, the median viral load for LruGHV1 in pumas was lower than that for FcaGHV1 in domestic cats and LruGHV1 in bobcats and trended lower than that for PcoGHV1 in pumas (Fig. 3B). Viral loads for all three viruses were below 10,000 viral genomes per million cells, except in one FcaGHV1-infected domestic cat and two LruGHV1-infected bobcats, which had viral loads of between 58,000 and 490,000 genomes per million cells (Fig. 3B).

Global Positioning System coordinates for the capture locations were available for most animals sampled, allowing us to map the locations of viral DNA-positive and -negative cats within each study site (Fig. 4). FcaGHV1 was detected in domestic cats at all study sites, with a higher prevalence in California (28%, CI = 17 to 41%) than in Florida (13%, CI = 4 to 27%) and Colorado (6%, CI = 1 to 14%) (Fig. 4). LruGHV1 was detected in bobcats in each of the states (California, 37% [CI = 21 to 56%]; Colorado, 25% [CI = 9 to 49%]), with the highest prevalence being in Florida bobcats (76%, CI = 56 to 91%) (Fig. 4). Pumas were positive for LruGHV1 at approximately one-third the percentage of positivity for bobcats within each state (Fig. 4; California, 13% [CI = 5 to 26%]; Colorado, 9% [CI = 2 to 22%]; Florida, 25% [CI = 7 to 53%]). In the Colorado Western Slope (WS) and Florida sites, LruGHV1-positive pumas tended to be those that were captured in close geographic association with LruGHV1-positive bobcats (Fig. 4). PcoGHV1 was detected in only five pumas, all located within the San Diego and Riverside Counties (SDRC) study site northeast of San Diego, CA (Fig. 4; California, 13% [CI = 5 to

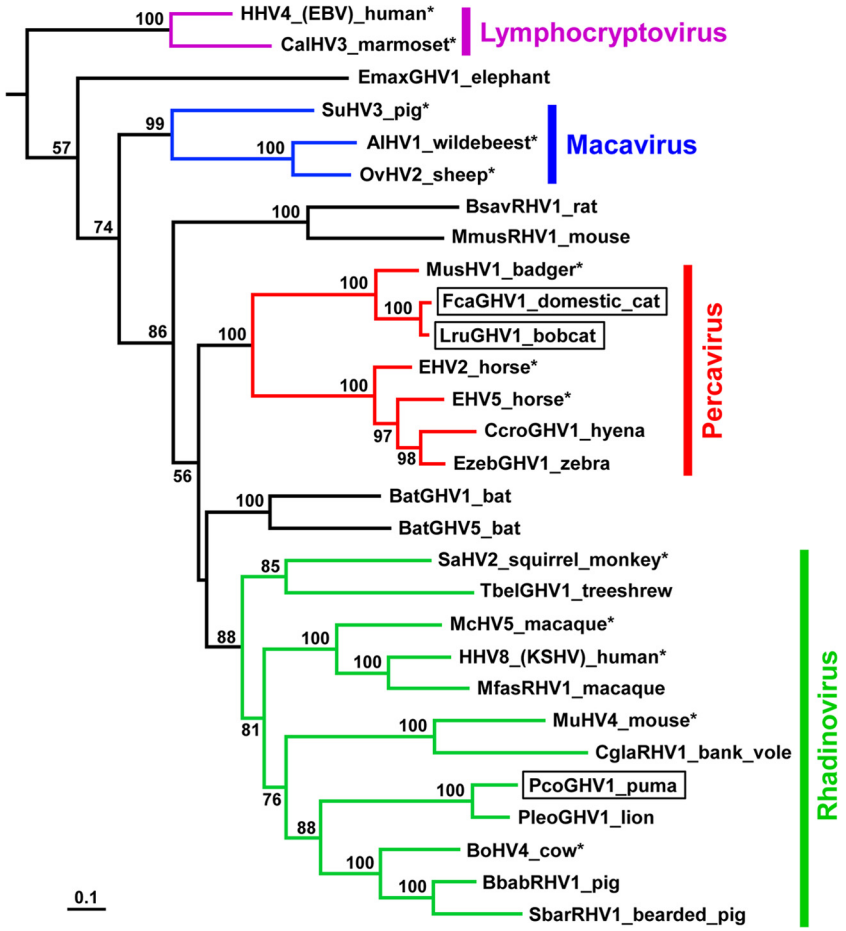

FIG 2 Maximum-likelihood phylogenetic analysis of gammaherpesviruses using concatenated DNApol and gB amino alignments. The betaherpesvirus human cytomegalovirus (HHV5; GenBank accession no. NC006273) was used as an outgroup to root the tree but is not displayed due to space constraints. Phylogenetic clusters corresponding to GHV genera were inferred on the basis of the most recent common ancestor of recognized members of each GHV genus (indicated with asterisks) and are shown in color. Bootstrap support out of 100 replicates is displayed for each node (values of <50 are not shown). Virus abbreviations, their definitions, and the GenBank accession numbers are as follows: HHV4, human herpesvirus 4 (Epstein-Barr virus), NC007605; CalHV3, callitrichine herpesvirus 3, NC004367; EmaxGHV1, *Elephas maximus* gammaherpesvirus 1, EU085379; SuHV3, suid herpesvirus 3, AF478169; AlHV1, alcelaphine herpesvirus 1, NC002531; OvHV2, ovine herpesvirus 2, NC007646; BsavRHV1, *Bandicota savilei* rhadinovirus 1, DQ821581; MmusRHV1, *Mus musculus* rhadinovirus 1, AY854167; MusHV1, mustelid herpesvirus 1, AF376034; FcaGHV1, *Felis catus* gammaherpesvirus 1, KF840715; LruGHV1, *Lynx rufus* gammaherpesvirus 1, KF840716; EHV2, equid herpesvirus 2, NC001650; EHV5, equid herpesvirus 5, AF050671; CcroGHV1, *Crocuta crocuta* gammaherpesvirus 1, DQ789371; EzebGHV1, *Equus zebra* gammaherpesvirus 1, AY495965; BatGHV1, bat gammaherpesvirus 1, DQ788623; BatGHV5, bat gammaherpesvirus 5, DQ788629; SaHV2, saimiriine herpesvirus 2, NC001350; TbelGHV1, *Tupaia belangeri* gammaherpesvirus 1, AY197561; McHV5, macacine HV5, NC003401; HHV8, human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus), NC009333; MfasRHV1, *Macaca fascicularis* rhadinovirus 1, AY138583; MuHV4, murid herpesvirus 4, NC001826; CglaRHV1, *Clethrionomys glareolus* rhadinovirus 1, AY854169; PcoGHV1, *Puma concolor* gammaherpesvirus 1, KF840717; PleoGHV1, *Panthera leo* gammaherpesvirus 1, DQ789370; BoHV4, bovine herpesvirus 4, NC002665; BbabRHV1, *Babyrousa babyrussa* rhadinovirus 1, AY177146; SbarRHV1, *Sus barbatus* rhadinovirus 1, AY177147.

26%]; SDRC, 24% [CI = 9 to 44%]). One of these pumas was also found to be coinfected with LruGHV1 (Fig. 4).

Risk factors for feline gammaherpesvirus infection. We examined how the probability of GHV infection, as detected by qPCR of blood cell DNA, was related to host sex, age, state, and site within state (Tables 3 and 4). We detected significant effects of sex, age, and state for domestic cat FcaGHV1, age and state for bobcat LruGHV1, and state and site for puma PcoGHV1 (Table 4). We then calculated individual odds ratios for all pairwise comparisons within each predictor (Table 5). All 21 of the FcaGHV1-positive domestic cats were adult males (Table 3), resulting in odds ratios of 21.8 for infection in males versus females and 8.2 for infection in adult cats versus young cats (Table 5). FcaGHV1 occurred at a significantly higher prevalence in California than Colorado (Table 5). Adult bobcats exhibited a higher probability of LruGHV1 infection than young bobcats, and bobcats in Florida had a higher probability of infection than bobcats in California and Colorado (Table 5). Host sex or age was not predictive of puma infection with LruGHV1 or PcoGHV1 (Table 5). The small number of puma samples positive for PcoGHV1 or LruGHV1 diminished the capacity to detect effects of sex, age, state, and site as risk factors for puma infection.

DISCUSSION

In this study, we identified nucleotide sequences in blood cell DNA from three novel GHVs in three felid hosts. These sequences

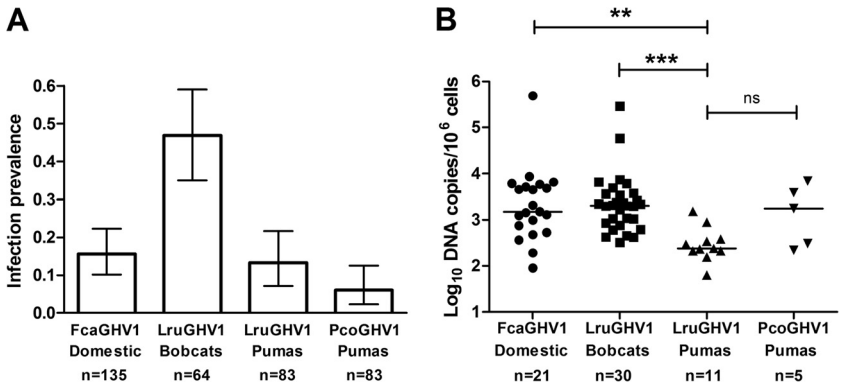

FIG 3 Prevalence and DNA viral loads of gammaherpesviruses in felid species. (A) Prevalence of infection was determined by qPCR on blood cell DNA. Domestic cat samples were tested for FcaGHV1. Bobcat and puma samples were tested for LruGHV1 and PcoGHV1. All bobcat samples were negative for PcoGHV1 ($n = 64$; data not shown). (B) Viral load was determined for samples testing positive by qPCR. Lines, median viral loads. Statistical significance is indicated graphically: **, $P < 0.01$; ***, $P < 0.001$; ns, not significant ($P > 0.05$).

represented ~3.4 kb across two conserved herpesviral genes, providing strong evidence for the existence of novel feline GHVs. We assigned provisional names to these viruses according to their apparent primary host species: *Felis catus* GHV1 (FcaGHV1) in domestic cats, *Lynx rufus* GHV1 (LruGHV1) in bobcats, and *Puma concolor* GHV1 (PcoGHV1) in pumas. LruGHV1 was also detected at a lower prevalence and with a lower viral load in pumas than in bobcats, suggestive of ongoing bobcat-to-puma transmission. Viral loads were low for all three viruses (<10,000 genomes per million cells; Fig. 3B), with several outliers with high viral loads, which is consistent with a predominance of latency with rare active replication. The risk of infection varied with geographic location for each host species: domestic cats in California had the highest prevalence of FcaGHV1, bobcats in Florida had the highest prevalence of LruGHV1, and only pumas from one California study site were positive for PcoGHV1. For domestic cats and bobcats, adult age was a risk factor for GHV infection, while being male was an infection risk factor for domestic cats.

The sequences of FcaGHV1 and LruGHV1 are closely related, with 92% nucleotide identity across the 3.4-kb region sequenced

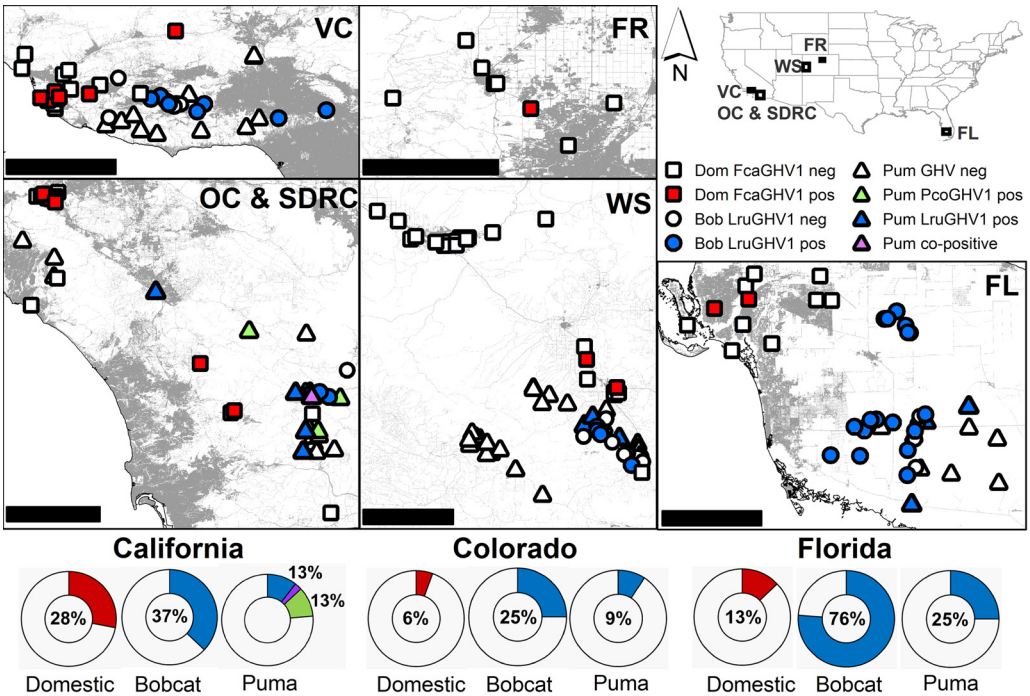

FIG 4 Geographic distribution of GHV-positive and -negative felids. Domestic cats (Dom), bobcats (Bob,) and pumas (Pum) from six study sites in three states are displayed with symbols at the location of capture. Host species is indicated by symbol shape, and viral species is indicated by symbol color. The copositive puma was positive for both LruGHV1 and PcoGHV1. Study sites are abbreviated as follows: VC, Ventura County, CA; OC, Orange County, CA; SDRC, San Diego and Riverside Counties, CA; FR, Front Range, CO; WS, Western Slope, CO; FL, Florida. Circle graphs display the prevalence of qPCR-positive samples within each state for each virus-host relationship. Shaded areas represent impervious surfaces indicative of urban and developed landscapes (e.g., asphalt, concrete, and highly compacted soils) derived from the National Land Cover Database (61).

TABLE 3 Numbers of GHV qPCR-negative and -positive individuals for each predictor (sex, age, state, and site)[a]

	Domestic cat (FcaGHV1)			Bobcat (LruGHV1)			Puma					
							LruGHV1			PcoGHV1		
	No of samples			No of samples			No of samples			No of samples		
Predictor	Neg	Pos	%	Neg	Pos	%	Neg	Pos	%	Neg	Pos	%
Sex												
Female	54	0	0	15	10	40.0	31	7	18.4	36	2	5.3
Male	51	21	29.2	19	20	51.3	40	4	9.1	41	3	6.8
Age												
Young	30	0	0	9	2	18.2	17	2	10.5	18	1	5.3
Adult	76	21	21.6	25	28	52.8	48	9	15.8	54	3	5.3
State												
California	36	14	28.0	17	10	37.0	33	5	13.2	33	5	13.2
Colorado	51	3	5.6	12	4	25.0	30	3	9.1	33	0	0
Florida	27	4	12.9	5	16	76.2	9	3	25.0	12	0	0
Site[b]												
VC	11	6	35.3	15	7	31.8	15	0	0	15	0	0
OC	11	6	35.3	—	—	—	2	0	0	2	0	0
SDRC	14	2	12.5	2	3	60.0	16	5	23.8	16	5	23.8
WS	43	2	4.4	12	4	25.0	30	3	9.1	33	0	0
FR	8	1	11.1	—	—	—	—	—	—	—	—	—

[a] FcaGHV1 was identified in domestic cats, LruGHV1 was identified in bobcats and pumas, and PcoGHV1 was identified in pumas. All samples had location information, but several samples did not have sex and/or age recorded. Neg, negative; Pos, positive; —, sites from which samples were not evaluated.

[b] Samples in Florida were considered to be from a single site and thus are only included in the state-by-state comparison and not in the site-by-site comparison.

in this study. Suid herpesviruses 3 and 4 (porcine lymphotropic herpesviruses 1 and 2, respectively) have 93% nucleotide identity across the same genetic region (GenBank accession no. AF478169 and AY170314, respectively) and are considered separate viral species (37). By comparison, this indicates that FcaGHV1 and LruGHV1 constitute different viral species (rather than strains of the same species). The amino acid sequences of gB and DNApol of FcaGHV1 and LruGHV1 were phylogenetically related to those of other GHVs in the *Percavirus* genus, while those of PcoGHV1 were more closely related to those of viruses within the *Rhadinovirus* genus, particularly PleoGHV1 of African lions. Thus, the two clusters of contemporary felid GHVs share more common ancestry with viruses of nonfelid hosts than with each other. Furthermore, the relationships among the felid GHVs are not concordant with the phylogeny of felid host species. For instance, while the puma virus (PcoGHV1) and lion virus (PleoGHV1) are closely related, pumas share a more recent common ancestor with bobcats and domestic cats than lions (11). Therefore, we conclude that these felid GHVs evolved from at least two distinct lineages, which may have originated from viruses of nonfelid hosts. To our knowledge, these are the first GHVs identified to be native to domestic cats, bobcats, and pumas. Identification of novel GHVs in all three species indicates that GHV infection of felids may be common, despite the lack of published reports of such agents.

The qPCR data presented in this study provide useful estimates of felid GHV infection prevalence but may underestimate actual prevalence. The ability of GHVs to establish lifelong latent infection can lead to persistence of the virus at very low levels in some hosts (3) that may not have been detected in these assays. Many animals in this study population had less than one GHV DNA genome detected per 1,000 cells ($<10^3$ copies per million cells; Fig. 3B), suggesting that some infections could remain undetected. Furthermore, while GHVs are typically lymphotropic (4, 5), it is possible that tissue reservoirs of virus which would be missed exist because only blood cell DNA was sampled. Assays to detect the presence of antibodies to these viruses would be useful for comparing the prevalence of detectable infection (DNA) to evidence of virus exposure (antibodies). Further, evidence of viral replication (i.e., culturing of virus *ex vivo* or detection of viral particles or viral RNA) is required to conclusively document the infectious capacity of these agents. Nevertheless, the use of GHV-specific qPCR assays to screen felid DNAs provided insight into the relative frequency of infection, virus geographic distribution, and risk factors for infection, such as the strong bias of FcaGHV1 for adult male cats.

LruGHV1 infection of bobcats had the highest prevalence (47%, 30 of 64 samples), followed by FcaGHV1 infection of domestic cats (16%, 21 of 135 samples), LruGHV1 infection of pumas (13%, 11 of 83 samples), and PcoGHV1 infection of pumas (6%, 5 of 83 samples) (Fig. 3A). The higher GHV prevalence in bobcats relative to the prevalence of either of the GHVs in pumas may reflect the ~2- to 10-fold greater densities of bobcats than pumas (43) and, potentially, density-associated contact and transmission rates. However, domestic cats have higher densities than bobcats (44) yet a lower GHV prevalence. Therefore, GHV prevalence may be influenced by additional factors, such as viral and host biologic factors, including route of transmission, infectious dose, and blood cell infection rates.

Geographically, FcaGHV1 and LruGHV1 were found at all study sites (Fig. 4 and Table 3), suggesting that these viruses are widespread in the United States. In contrast, PcoGHV1 was found

TABLE 4 Effects of sex, age, state, and site on GHV infection status of domestic cats, bobcats, and pumas[a]

	Domestic cat (FcaGHV1)			Bobcat (LruGHV1)			Puma					
							LruGHV1			PcoGHV1		
Characteristic	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*
Sex	23.636	1	**<0.001**	0.932	1	0.338	1.155	1	0.286	0.003	1	0.955
Age	6.158	1	**0.015**	4.735	1	**0.034**	0.13	1	0.72	<0.001	1	0.991
State	3.53	2	**0.033**	6.229	2	**0.004**	1.218	2	0.302	3.374	2	**0.04**
Site[b]	0.815	3	0.488	0.478	1	0.492	1.779	2	0.177	3.552	2	**0.034**
Residual		116			58			69			69	

[a] FcaGHV1 was identified in domestic cats, LruGHV1 was identified in bobcats and pumas, and PcoGHV1 was identified in pumas. Significant relationships are indicated in bold. df, degrees of freedom.

[b] Site nested within state for analyses.

TABLE 5 Individual odds ratios for predictor variables of GHV infection[h]

	Odds ratio (CI)			
			Puma	
Comparison group	Domestic cat (FcaGHV1)	Bobcat (LruGHV1)	LruGHV1	PcoGHV1
Male vs female	**21.808 (2.686–770.797)**	1.364 (0.571–4.214)	0.378 (0.133–1.642)	0.857 (0.229–6.626)
Adult vs young	**8.182 (1.007–291.993)**	**4.685 (1.053–36.205)**	1.041 (0.307–6.124)	0.491 (0.109–5.760)
California vs Colorado	**4.824 (1.688–20.238)**	1.333 (0.445–6.248)	1.103 (0.343–5.961)	**1.141 (1.012–1.285)**
California vs Florida	2.043 (0.755–7.804)	**0.163 (0.058–0.685)**[a]	0.331 (0.097–2.041)	1.141 (0.935–1.392)[b]
Florida vs Colorado	1.821 (0.553–10.488)	**6.400 (1.961–35.400)**	2.250 (0.616–16.732)	—[c]
VC vs OC	0.786 (0.257–3.897)	—[d]	—[e]	—[e]
SDRC vs VC	2.333 (0.629–17.091)	0.219 (0.055–2.181)	**1.269 (1.012–1.591)**	**1.269 (1.012–1.591)**
SDRC vs OC	2.333 (0.629–17.091)	—[d]	1.269 (0.676–2.383)[f]	1.269 (0.676–2.383)[f]
WS vs FR	1.593 (0.356–26.454)	—[g]	—[g]	—[g]

[a] For the reciprocal comparison, Florida versus California, the odds ratio is 4.121 (CI = 1.459 to 17.132).
[b] Even though no infected individuals were detected in Florida, confidence intervals overlap 1 due to the small number of infected individuals from California ($n = 4$) and the relatively small number of samples from Florida ($n = 12$).
[c] —, no infected individuals were from Colorado or Florida.
[d] —, no samples from OC were tested.
[e] —, no positive samples were from VC or OC.
[f] Even though no infected individuals were detected at OC, confidence intervals overlap 1 due to the small number of samples from OC ($n = 2$).
[g] —, no samples from FR were tested.
[h] FcaGHV1 was identified in domestic cats, LruGHV1 was identified in bobcats and pumas, and PcoGHV1 was identified in pumas. Significant relationships are indicated in bold.

only in pumas in the study site in San Diego and Riverside Counties, CA, at a 24% within-site prevalence (Fig. 4 and Table 3). This was especially striking, since all 15 pumas in nearby Ventura County, CA, north of Los Angeles, tested negative (Fig. 4 and Table 3). The close phylogenetic relationship of PcoGHV1 to PleoGHV1 of the African lion suggests a felid host origin rather than recent introduction from another nonfelid species, such as a puma prey species. Testing of pumas from Mexico and Central and South America would help elucidate if the range of this virus extends southward. The close relationship between FcaGHV1 and LruGHV1 may indicate a more recent cross-species transmission event or similar evolutionary pressures exerted by hosts on these viruses. Evaluation of GHV sequences in other carnivores, particularly domestic and wild felids, may further elucidate the evolutionary history and host range of GHVs.

The detection of identical LruGHV1 sequences in bobcats and pumas is particularly intriguing. The 3- to 4-fold higher prevalence of this virus in bobcats than pumas (Fig. 3A) suggests that the bobcat is the primary host. In addition, the LruGHV1 load in pumas was significantly lower than the load of LruGHV1 in bobcats and the load of FcaGHV1 in domestic cats (Fig. 3B), suggesting that LruGHV1 may be poorly adapted for pumas. Pumas are known to occasionally kill bobcats (45), which suggests a potential mechanism for bobcat-to-puma cross-species transmission. The distribution of LruGHV1 in pumas has striking similarity to the distribution of the bobcat feline immunodeficiency virus (FIV) strain FIV-PcoA in pumas. FIV-PcoA is found in California and Florida bobcats but also infects a lower percentage of pumas in these locations (46–48). In contrast, the FIV-PcoB strain infects only pumas, similar to PcoGHV1 (49, 50). It remains to be determined whether unique aspects of the dynamics of viral transmission or species interaction patterns in these regions underlie this similarity. Future analyses assessing correlations between the presence of FIVs and GHVs in individual animals may shed light on potential relationships. It is possible that a third species, such as a common prey species, could transmit LruGHV1 to both bobcats and pumas. However, the genetic similarity between LruGHV1 and other percaviruses of diverse carnivores, such as sea otters (41), which do not share prey species with felids, suggests a carnivore host. The finding of a single GHV in two related species serves as a caution against making strong assumptions about GHV host species relationships on the basis of findings for a very small number of samples.

Comparison of GHV infection, as detected by qPCR, to cat capture location, age, and sex yielded several notable relationships. The strong bias for detection of FcaGHV1 in adult male domestic cats is particularly compelling and suggests a strong link between FcaGHV1 exposure and male cat behavior. A higher prevalence in adult animals than young animals was also found for LruGHV1-infected bobcats and is indicative of an enhanced infection risk with age, consistent with our observations of other chronic infections in wild and domestic cats (19), and is suggestive of horizontal versus vertical transmission. The strong sex bias observed for FcaGHV1 was not observed for the GHVs of wild cats, and the basis for this finding in the feral domestic cat population is unclear. Ecologically, domestic cats are relatively divergent from wild felids. Higher densities and the communal behavior of domestic cats, relative to wild cats, could lead to more intraspecific aggressive encounters among males (51, 52). Male domestic cats may also defend territories more rigorously than female domestic cats, whereas in the wild felids, territories may be defended by both sexes (52–54). Biologic factors, such as hormonal differences, could also potentially influence host immunity or viral reactivation and replication (55).

To our knowledge, FcaGHV1 is the first GHV identified to be native to domestic cats. Domestic cats are important companion animals present in millions of homes worldwide. Given the role of EBV and KSHV in causing cancer in HIV-infected humans (7–9, 56), it will be highly relevant to determine replication patterns and pathogen characteristics of FcaGHV1 to determine whether a link exists between FIV-associated lymphoma and FcaGHV1. In addition, cats are prone to lymphoproliferative disorders, including solid tissue lymphomas, leukemias, and lymphocytoses (57–60), which could potentially have a GHV etiology. Further research on

this interesting and apparently widespread potential pathogen is warranted.

Identification of these felid GHVs expands our understanding of herpesvirus diversity and phylogeny. Furthermore, GHV qPCR screening of numerous felid DNA samples from study sites in three different U.S. states contributes valuable information on the biology of these viruses, including their prevalence, geographic distribution, and host range, and the host characteristics associated with infection. This information has the potential to inform both feline health and virus ecology.

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