

Short Communication

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Molecular identification of a novel gammaherpesvirus in the endangered Darwin's fox (*Lycalopex fulvipes*)

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We report the detection and characterization of a novel gammaherpesvirus in the critically endangered Darwin's fox (*Lycalopex fulvipes*; syn. *Pseudalopex fulvipes*) on Chiloé Island, Chile. Out of 28 analysed blood samples stored in alcohol, four were positive for this herpesvirus using a previously described pan-herpesvirus PCR assay targeting the herpesvirus DNA polymerase. Positive samples were subsequently characterized by means of a PCR targeting a 500 bp fragment of the glycoprotein B of the gammaherpesviruses. This novel herpesvirus was most closely related to other gammaherpesviruses from terrestrial carnivores, and is tentatively named Darwin's fox gammaherpesvirus (canid herpesvirus 2). No apparent lesions were observed in the surveyed foxes. This is the first report of a gammaherpesvirus infecting a canid worldwide, and also of one infecting a carnivore from South America.

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Received 31 July 2013

Accepted 12 September 2013

The order *Herpesvirales* is a vast order of currently approximately 130 large, enveloped DNA virus species divided into three families. The family *Herpesviridae* contains approximately 79 known virus species of mammals and is further subdivided into three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* (Widén *et al.*, 2012). Gammaherpesviruses usually have a host range restricted to the host's family or order. So far, gammaherpesvirus infection has been reported in only five species of terrestrial carnivores: the free living European badger (*Meles meles*) and Northern sea otter (*Enhydra lutris kenyoni*), the lion (*Panthera leo*), and in a captive fisher (*Martes pennanti*) and an oriental small-clawed otter (*Aonyx cinerea*) (Table 1).

Viruses of this subfamily have specificity for either B- or T-lymphocytes and may cause lymphoproliferative disease.

The GenBank/EMBL/DDBJ accession numbers for the polymerase and glycoprotein B sequences of canid herpesvirus 2 are HF586888 and KF471019, respectively.

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Latency of gammaherpesviruses may be established in lymphoid tissue. Infections with viruses from this subfamily generally cause few clinical signs in the main host but may cause severe disease in other related species (Widén *et al.*, 2012). Gammaherpesvirus infection has been associated with skin and mucosal ulcerative lesions in one fisher (Gagnon *et al.*, 2011) and in sea otters (Tseng *et al.*, 2012) but also in marine members of the order Carnivora (e.g. Goldstein *et al.*, 2006).

The aim of the present communication was to report the identification of a novel member of the subfamily *Gammaherpesvirinae* infecting Darwin's fox (*Lycalopex fulvipes*; syn. *Pseudalopex fulvipes*). This species is considered critically endangered by the International Union for Conservation of Nature (IUCN) and is one of the most endangered carnivores worldwide with only about 250 individuals remaining (Jiménez *et al.*, 2012). Information about its susceptibility to infectious agents is almost non-existent.

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Table 1. Summary of herpesviruses reported to date in wild terrestrial carnivores

Host	Herpesvirus	GenBank accession no.	Lesions attributable to herpesvirus	Prevalence by PCR	Location	Reference(s)
Fisher (<i>Martes pennanti</i>)*	FiHV	HM579931	Multiple skin ulcers on the muzzle and plantar pads†	Single case	Canada	Gagnon <i>et al.</i> (2011)
Oriental small-clawed otter1 (<i>Aonyx cinerea</i>)	Oriental small-clawed otter herpesvirus	FJ797657	NR	Single case	Hungary	Janoska and Harrach, unpublished data
European badger (<i>Meles meles</i>)	Badger herpesvirus (BadHV)‡	AF376034	Pulmonary fibroblast in one individual	96 % of 29	UK and Ireland	Banks <i>et al.</i> (2002); King <i>et al.</i> (2004)
Northern sea otters (<i>Enhydra lutris kenyoni</i>)	Mustelid herpesvirus 1 (MusHV-1)‡ MusHV-2	GU799569 GU979535	NR Ulcerative lesions and plaques on the lingual, gingival, oral, oesophageal, and labial mucosa	Single case 46 % of 28 tissues and 34 % of 62 nasal swabs	Hungary Alaska, USA	Dandár <i>et al.</i> (2010) Tseng <i>et al.</i> (2012)
Lion (<i>Panthera leo</i>)	<i>Panthera leo</i> gammaherpesvirus 1	DQ789370	NR	Single case	NR	Ehlers <i>et al.</i> (2008)
Darwin's fox (<i>Pseudalopex fulvipes</i>)	Darwin's fox gammaherpesvirus	HF586888, KF471019	External lesions not observed	14 % of 28§	Chiloé Island, Chile	Present study

NR, Not reported.

*Captive born.

†It was not further confirmed whether the herpesvirus isolated was responsible for the observed lesions.

‡BadHV and MusHV-1 probably correspond to the same species.

§The storage of samples in alcohol probably resulted in an underestimation of the true prevalence. See text for details.

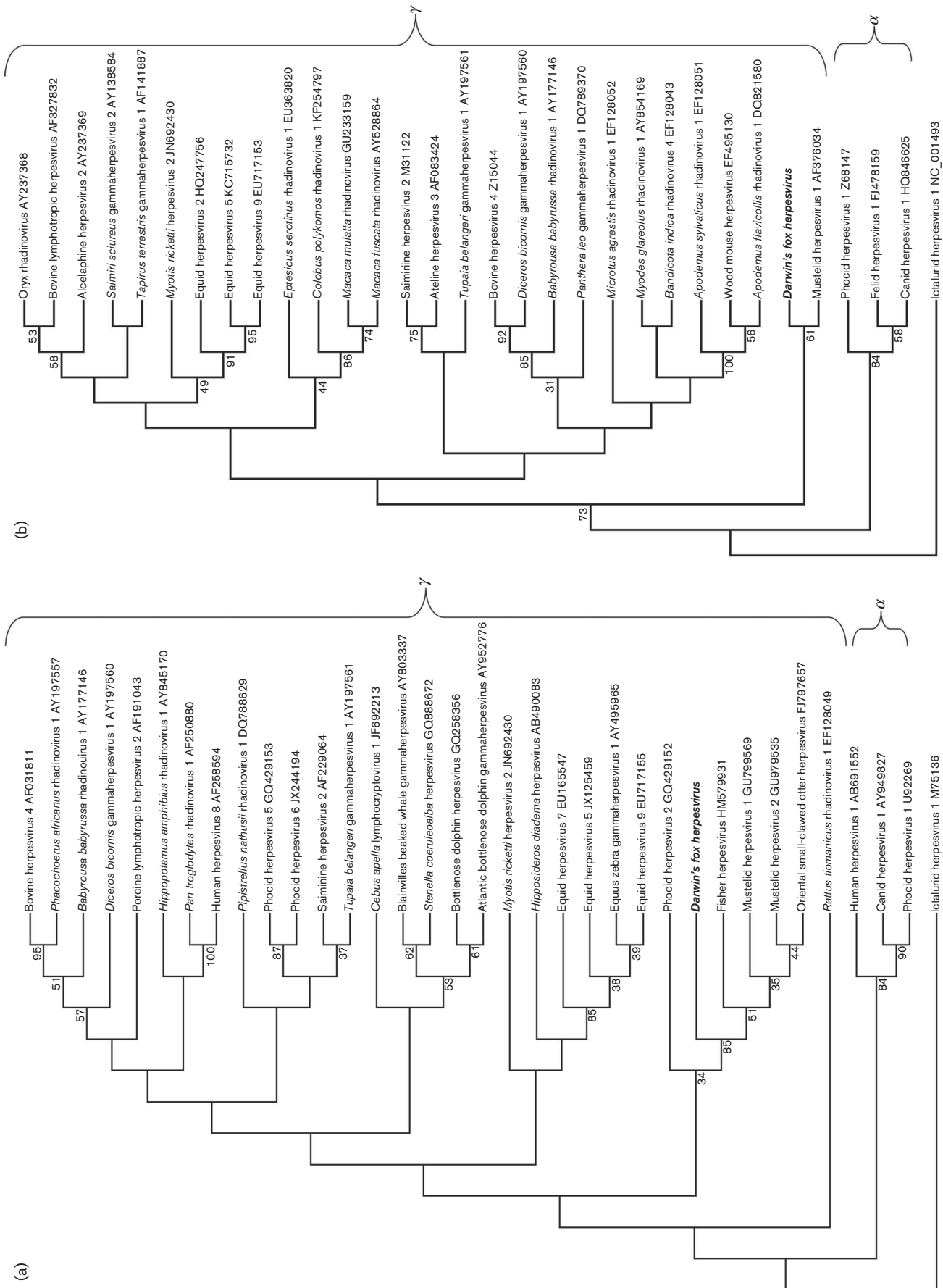


Fig. 1. Maximum-likelihood phylograms based on amino acid sequences of the herpesvirus DNA polymerase (a) and herpesvirus glycoprotein B (b). The bootstrap consensus tree was inferred from 1000 replicates generated with a random seed (MEGA5.2). The ictalurid herpesvirus 1 is included as an outgroup member. The name of each sequence indicates the host species followed by the GenBank accession number. The GenBank accession numbers of Darwin's fox herpesvirus (canid herpesvirus 2) are HF586888 and KF471019 for the polymerase and glycoprotein B genes, respectively.

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Twenty-eight stored blood samples preserved in alcohol, collected between 2009 and 2012 during a fox population genetic study in Chiloé Island (41°46'S 74°00'W) were used. The samples consisted of 23 foxes older than 1 year (nine males and 14 females) and five foxes younger than 1 year (two males, three females). One adult female and three pups belonged to the same litter. Foxes were captured at 10 different sites on Chiloé with box traps. Traps were activated in the evening and revisited the next morning at dawn. Individuals were anaesthetized with a combination of 10 mg ketamine (Imalgene) kg⁻¹ and 1 mg xylazine (Xilacina) kg⁻¹. Approximately 200 µl blood collected from the cephalic vein was added to 1000 µl absolute ethanol. Foxes were clinically assessed by a veterinarian and released after full recovery at the capture site.

A portion of the blood sample (0.5 ml) was centrifuged and the pellet was washed three times. Total nucleic acid was extracted by pressure filtration (QuickGene DNA tissue kit S; FujiFilm Life Science) after a lysis step with lysis buffer (Cell Signaling Technology). Herpesvirus DNA detection was conducted using a pan-herpesvirus PCR

assay described previously (VanDevanter *et al.*, 1996). Direct sequencing was carried out with primers TGVseq and IYGseq to obtain a sequence fragment of approximately 167 bp, excluding primers. Positive samples were subsequently characterized molecularly, by means of the partial amplification of the glycoprotein B gene. A universal nested PCR that amplified a 500 bp fragment of the glycoprotein B gene of the gammaherpesviruses (GH1) was used (Ehlers *et al.*, 2008). DNA fragments were purified, cloned into the plasmid vector pGEM-T and grown in JM109 *Escherichia coli* following the manufacturer's instructions.

Amino acid *p*-distances were calculated prior to phylogenetic analysis. MEGA5.2 was used to construct phylogenetic trees for the polymerase and glycoprotein B proteins (Fig. 1) using the maximum-likelihood method based on amino acid sequences, with a bootstrap test of 1000 replicates.

Herpesvirus infection was confirmed in four foxes (14.3%; 95% confidence intervals 5.0–31.9). All the positive foxes were unrelated adults: two were captured in Chiloé National Park, located in the north-western part of the

Table 2. Estimation of evolutionary divergence between partial sequences of the herpesvirus polymerase gene

Lower left (bold): Number of amino acid differences per site from between sequences. Upper right: Number of nucleotide differences per site from between sequences. The analysis involved 12 sequences.

Virus (GenBank no.)	1	2	3	4	5	6	7	8	9	10	11	12
1. Darwin's fox herpesvirus (HF586888)		0.23	0.23	0.23	0.18	0.29	0.36	0.42	0.49	0.51	0.63	0.56
2. Fisher herpesvirus (HM579931)	0.22		0.10	0.08	0.15	0.32	0.41	0.41	0.49	0.51	0.64	0.58
3. Mustelid herpesvirus 1 (GU799569)	0.24	0.11		0.08	0.16	0.30	0.43	0.41	0.51	0.51	0.63	0.59
4. Mustelid herpesvirus 2 (GU979535)	0.28	0.11	0.09		0.13	0.33	0.45	0.41	0.53	0.54	0.64	0.59
5. Otter herpesvirus (FJ797657)	0.28	0.17	0.15	0.11		0.32	0.39	0.42	0.52	0.54	0.64	0.56
6. Phocid herpesvirus 2 (GQ429152)	0.37	0.39	0.39	0.41	0.41		0.42	0.44	0.55	0.53	0.59	0.59
7. Phocid herpesvirus 6 (JX244194)	0.41	0.43	0.43	0.46	0.43	0.52		0.22	0.53	0.53	0.65	0.60
8. Phocid herpesvirus 5 (GQ429153)	0.43	0.46	0.41	0.43	0.39	0.52	0.24		0.53	0.54	0.65	0.59
9. Phocid herpesvirus 1 (U92269)	0.63	0.61	0.63	0.63	0.63	0.63	0.67	0.63		0.23	0.53	0.74
10. Canid herpesvirus 1 (AY949827)	0.67	0.65	0.65	0.65	0.65	0.65	0.67	0.63	0.24		0.51	0.75
11. Human herpesvirus 1 (AB691552)	0.70	0.67	0.67	0.67	0.65	0.70	0.70	0.70	0.41	0.46		0.70
12. Ictalurid herpesvirus 1 (M75136)	0.80	0.80	0.83	0.85	0.83	0.83	0.85	0.87	0.96	0.96	0.89	

island, and the other two foxes were captured in Tantauco Park, a privately protected area in the south. This suggested that this agent is present throughout Chiloé Island.

The DNA polymerase gene fragment sequences from the four individuals identified a novel herpesvirus belonging to the subfamily *Gammaherpesvirinae* (Fig. 1), apparently most closely related to a group of mustelid herpesviruses, isolated from badger, otter species and a fisher from Canada (amino acid *p*-distance 0.217) and also closely related to other gammaherpesviruses of terrestrial carnivores (Fig. 1, Tables 1 and 2). Partial amplification of the glycoprotein B gene was successful, and the sequences obtained were also highly related to the mustelid herpesvirus 1 (MusHV-1) (amino acid *p*-distance 0.230). This agreed with previous observations suggesting that many elements in the branching patterns of members of the family *Herpesviridae* are congruent with branching patterns for the corresponding host species, being consistent with host–virus co-evolution (Wellehan *et al.*, 2008). As herpesviral species are named according to the family of the host species from which they was isolated (Davison *et al.*, 2009), we propose the tentative name of Darwin's fox gammaherpesvirus (canid herpesvirus 2).

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So far, gammaherpesvirus infection has been reported in only five species of carnivores, four of them belonging to the Mustelidae family (Table 1). This is thus the first report of a gammaherpesvirus infecting a canid worldwide, and the first infecting a carnivore from South America.

The pathogenicity of this novel virus remains unknown. No external lesions were observed in any of the animals. Herpesvirus infections are often latent and do not show lesions at the time of isolation (Banks *et al.*, 2002). For example, MusHV-1 has not so far been associated with lesions or clinical disease in badgers (King *et al.*, 2004). However, as mentioned above, lesions have indeed been found in some species. Also, many herpesvirus infections predispose the host to secondary bacterial infections and, taking into account the small population size and distribution area of Darwin's fox, we believe that this virus should be taken into consideration when implementing conservation strategies, such as translocation of individuals.

Acknowledgements

Foxes were captured with permission from the Servicio Agrícola y Ganadero de Chile (SAG) with permit numbers 1262/2009, 2262/2010 and 206/2012. Samples were exported and imported with CITES permits. We wish to thank Corporación Nacional Forestal (CONAF) and Tantauco Park for allowing us to capture foxes inside the protected areas and for their help in transport, accommodation and support on the ground; Tantauco Park for a grant awarded to J. C. to partially support the field work in 2009; Cooperativa de Pescadores Mar Adentro, for maritime transport and accommodation in Ahuenco; and J. Hetz, M. Mora, V. Sánchez, T. Vuskovic, V. Solé and R. Palou, V. Nogal and E. Neves for their assistance in the field or in the laboratory. C. N. was supported by a doctoral fellowship from

the Instituto de Ecología y Biodiversidad (Facultad de Ciencias, Universidad de Chile) (ICM P05-002), Panthera Kaplan Awards Program (Panthera Foundation, NY, USA), Scott Neotropical Fund Award (Cleveland Metroparks Zoo & the Cleveland Zoological Society, Cleveland, USA) and the Eric York Scholarship (Felidae Conservation Fund, California, USA). J.M. holds a Ramón y Cajal contract awarded by the Ministerio de Ciencia e Innovación and the European Social Fund.

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