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Research article

Phylogenetic analysis of marine mammal herpesviruses

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ABSTRACT

Five novel DNA-dependent DNA polymerase (Dpol) herpesviral sequences were generated using nested consensus polymerase chain reaction (PCR) in clinical samples from a harbor seal (*Phoca vitulina*), bottlenose dolphin (*Tursiops truncatus*), orca (*Orcinus orca*), California sea lion (*Zalophus californianus*), and a *Phocid herpesvirus 2* (PhHV-2) isolate from a harbor seal (used as positive control). These novel sequences and other representative herpesvirus sequences were included in Bayesian and Maximum Likelihood analyses to illustrate the phylogeny of herpesviruses amongst the marine mammal host species and in comparison to those of other animals. All 19 novel and known marine mammal herpesviruses included in the analyses aligned with members of the *Alphaherpesvirinae* or *Gammaherpesvirinae* subfamilies. The novel harbor seal herpesvirus clustered with members of the *Macavirus genus*, subfamily *Gammaherpesvirinae*. The novel bottlenose dolphin herpesvirus clustered together in a monophyletic group with another delphinid alphaherpesvirus but could not be associated with an established genus. The orca herpesvirus also clustered with a delphinid alphaherpesvirus and formed a separate clade. The sea lion herpesvirus clustered with PhHV-2. PhHV-1 clustered with varicelloviruses and PhHV-2 clustered strongly in the *Gammaherpesvirinae* genus *Percavirus*. All cetacean gammaherpesviruses formed a monophyletic clade and could not be associated with an established gammaherpesviral genus.

1. Introduction

Most vertebrate species investigated have at least one, and usually several, endemic herpesviruses (Pellet and Roizman, 2007). More than 200 herpesvirus species have been identified to date (Pellet and Roizman, 2007). When

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investigating the lineage of herpesviruses, many major subdivisions mirror the phylogenetic branching order of their hosts (Pellet and Roizman, 2007). Thus, herpesviruses appear to have codiverged with their hosts and tend to be host specific (Pellet and Roizman, 2007). Since there are almost 5500 different species of mammals alone (Schipper et al., 2008), it can be expected that the number of herpesviruses that exist in nature well exceeds the 200 species identified thus far. All herpesviruses fall within the newly established taxonomic order *Herpesvirales* (Davison et al., 2009). *Herpesvirales* consists of three families: *Herpesviridae* (which includes the herpesviruses of mammals, reptiles, and birds), *Alloherpesviridae* (which includes the herpesviruses of fish and amphibians), and *Malacoherpesviridae* (bivalve herpesviruses) (Davison et al., 2009; Pellet and Roizman, 2007). Additionally, the family *Herpesviridae* contains three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae* (Davison et al., 2009; Pellet and Roizman, 2007).

The first marine mammal herpesvirus, *Phocid herpesvirus 1* (PhHV-1), was reported in 1985 from several harbor seals (*Phoca vitulina*) in The Netherlands with signs of acute pneumonia and hepatitis virus (Osterhaus et al., 1985). Electron microscopy (EM), serum neutralization, as well as other herpesvirus characteristics were used to identify the alphaherpesvirus in lung and liver isolates (Osterhaus et al., 1985). In 1994, a second herpesvirus, identified in harbor seal leukocytes, was reported (Lebich et al., 1994). Genetic analysis of phocid herpesvirus isolates in 1996 confirmed a novel gammaherpesvirus, PhHV-2 (Harder et al., 1996). In addition to reports in harbor seals, PhHV-1 has also been reported in a grey seal (*Halichoerus grypus*). A putative gammaherpesvirus isolate from a California sea lion (*Zalophus californianus*) was in fact from a grey seal but was mislabeled (Harder et al., 1996; Kennedy-Stoskopf et al., 1986; Kennedy-Stoskopf, 2001). Currently, one alphaherpesvirus (*Phocid herpesvirus 1*) and four gammaherpesviruses (*Phocid herpesvirus 2*, Hawaiian monk seal herpesvirus, Otarine herpesvirus 1, and Northern elephant seal herpesvirus) have been reported in pinnipeds (Goldstein et al., 2006a,b; Harder et al., 1996; Lebich et al., 1994; Lipscomb et al., 2000; Osterhaus et al., 1985).

The presence of herpesviruses in cetaceans (the group of animals consisting of all porpoises, dolphins, and whales) has been recognized since the late 1980s with electron microscopy (EM) reports of herpesvirus-like particles in skin biopsies from beluga whales (*Delphinapterus leucas*) (Barr et al., 1989; Martineau et al., 1988). Herpesviruses have also been associated with encephalitis in a harbor porpoise (*Phocoena phocoena*) (Kennedy et al., 1992) and skin lesions of dusky dolphins (*Lagenorhynchus obscures*) by EM (Van Bressem et al., 1994). In addition to EM, immunoperoxidase staining (Kennedy et al., 1992), serum neutralization and enzyme-labeled immunosorbent assays have been used as indicators for the presence of herpesviruses in cetaceans (Mikaelian et al., 1999). The first cetacean herpesviral genetic sequences, derived from stranded bottlenose dolphins (*Tursiops truncatus*), were not published until 2001 (Blanchard et al., 2001). Currently, four alphaherpesviruses and two gammaherpesviruses have been reported in bottlenose dolphins (Blanchard et al., 2001; Manire et al., 2006; Smolarek Benson et al., 2006). Herpesviral genomic sequence has also been reported from a Blainville's beaked whale (*Mesoplodon densirostris*), a dwarf sperm whale (*Kogia sima*), and a Risso's dolphin (*Grampus griseus*) (Smolarek Benson et al., 2006).

In this study, several novel herpesviruses were detected in cetacean and pinniped samples submitted by veterinarians as part of the diagnostic investigation of clinical cases. The relatedness of these partial, new herpesviral sequences to representatives of the known *Herpesviridae* subfamilies and genera was then explored through Bayesian and Maximum Likelihood (ML) analyses. Previous works focus on cetacean, pinniped, or sirenian herpesviruses and preclude a phylogenetic analysis of all marine mammal herpesviruses. This study takes a comprehensive approach by including distinct herpesviruses from both aquatic and terrestrial species.

2. Materials and methods

2.1. Animals/PCR amplification and sequencing

A mucosal swab from a harbor seal (*P. vitulina*), a buffy coat from a bottlenose dolphin (*T. truncatus*), blowhole exudate from an orca (*Orcinus orca*), an eye swab from a California sea lion (*Z. californianus*), and a *Phocid herpesvirus 2* isolate from a harbor seal (used as positive control) were collected. DNA was extracted via a commercial kit according to manufacturer's instructions (DNeasy Blood and Tissue Kit, Qiagen Inc., Valencia, CA). PCR was performed on all samples with nested consensus primers for the DNA-dependent-DNA polymerase (Dpol) of herpesviruses (VanDevanter et al., 1996). Additional forward primers were designed (SIIQfor [5′-AGY ATH ATH CAR GCN CAY AA-3′] and DIEC [5′-KND SNT TYG AYA THG ART G-3′]) for use with the IYG reverse consensus primer (VanDevanter et al., 1996) to extend the partial Dpol sequence. Reactions were amplified in a thermal cycler (Px2, Thermo Fisher Scientific, Inc., Waltham, MA) using Platinum® *Taq* DNA Polymerase (Invitrogen Corp., Carlsbad, CA) in 30 μL reactions following manufacturer's instructions. Amplification conditions were as follows: initial denaturation at 94 °C for 5 min; 45 cycles of amplification with each cycle consisting of denaturation at 94 °C for 30 s, annealing at 46 °C for 1 min, and elongation at 72 °C for 1 min; a final elongation step was performed at 72 °C for 7 min followed by a 4 °C hold. Secondary PCR products were resolved on a 1–1.5% agarose gels. Bands of expected size (TGV/IYG: 170–315 bp; SIIQ/IYG: 438–456 bp; DIEC/IYG: 1308 bp) were excised and purified using a QIAquick Gel Extraction Kit (Qiagen Inc.). Direct sequencing was performed (BigDye Terminator v3.1 Kit, Applied Biosystems Inc., Foster City, CA) using the second round primers, TGV and IYG (VanDevanter et al., 1996), and/or the extended primers, SIIQ and DIEC. Sequencing reactions were submitted to the University of Florida Interdisciplinary Center for Biotechnology Research and analyzed on ABI 3130 DNA sequencers (Applied Biosystems Inc.). Primer sequences were edited out prior to further analysis. Sequences were confirmed via translated nucleotide-translated nucleotide and protein BLAST search in the

National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2. Phylogenetic analysis

Forty-five predicted homologous amino acid Dpol sequences (ranging 144–152 in length), including fourteen marine mammal herpesviruses, were aligned using three methods: ClustalW2 (Larkin et al., 2007), T-Coffee (Notredame et al., 2000), and MUSCLE (Edgar, 2004). Bayesian analysis of each alignment was performed with MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) with gamma-distributed rate variation plus a proportion of invariant sites, and mixed amino acid substitution models. Four chains were run and statistical convergence was assessed by looking at the standard deviation of split frequencies as well as potential scale reduction factors of parameters. The first 10% of 1,000,000 iterations were discarded as a burn-in. *Iguanid herpesvirus 2* (GenBank accession number AY236869) was designated as the outgroup due to its early divergence from other herpesviruses (McGeoch and Gatherer, 2005; Wellehan et al., 2003).

Two separate analyses were also run for smaller amino acid regions of alpha- and gammaherpesviruses (58–64 and 55–60, respectively). These analyses were added to allow for the inclusion of four additional marine mammal herpesviruses. MUSCLE was used to align the 19 alphaherpesvirus and 20 gammaherpesvirus predicted homologous amino acid Dpol sequences. *Iguanid herpesvirus 2* was designated as the outgroup for the alphaherpesvirus alignment and *Elephantid herpesvirus 1* (GenBank accession number AF322977) was designated as the outgroup for the gammaherpesvirus alignment.

Protein maximum likelihood (ProML) analyses of each amino acid alignment was performed using PHYLIP (Phylogeny Inference Package, Version 3.66) (Felsenstein, 1989) with amino acid substitution models Jones–Taylor–Thornton (JTT) (Jones et al., 1992), Point Accepted Mutation (PAM) (Kosiol and Goldman, 2005), and Probability Matrix from Blocks (PMB) (Veerassamy et al., 2003). All models were set for global rearrangements, five replications of random input order, less rough, gamma plus invariant rate distributions, and unrooted. The value for the alpha of the gamma distribution was taken from the Bayesian analysis and the proportion of invariant sites was directly taken from the data set. The alignments were also used to create data subsets for bootstrap analysis to test the strength of the tree topology (100 re-samplings) which were analyzed using the amino acid substitution model producing the most likely tree (Felsenstein, 1985).

3. Results

3.1. Animals/PCR amplification and sequencing

For the purpose of clarity while discussing 19 marine mammal herpesviruses, previously reported pinniped and cetacean herpesviruses were tentatively named in accordance with the International Committee on Taxonomy of Viruses (ICTV) naming conventions for herpesviruses. The proposed names are derived from the host family and order of virus discovery (Davison et al., 2005, 2009) as demonstrated by the previously ICTV accepted names *Phocid herpesvirus 1* and *2*. References and GenBank accession numbers for the marine mammal herpesviruses are shown in Table 1.

Novel, genetically distinct, partial Dpol herpesviral sequences were detected in the harbor seal, bottlenose dolphin, orca, and California sea lion samples. Additionally, the bottlenose dolphin sample contained a second herpesvirus 100% identical, at the nucleotide level, to a previously described alphaherpesvirus (Delphinid HV 2; AF245443) in a stranded dolphin (Blanchard et al., 2001). Since the sequence reported herein is a larger segment of the Dpol region (444 bp) it was submitted to GenBank (accession number GQ429149) and used in the phylogenetic analysis. The other cDNA sequence from the

Table 1
Names of marine mammal herpesviruses and sources for the original polymerase sequences.

Name	Abbreviation	Species	Ref.	GenBank accession number
Delphinid HV 1	DeHV-1	*Tursiops truncatus*	Blanchard et al. (2001)	AF196646
Delphinid HV 2	DeHV-2	*Tursiops truncatus*	Blanchard et al. (2001)	AF245443
Delphinid HV 3	DeHV-3	*Tursiops truncatus*	Manire et al. (2006)	AY757301
Delphinid HV 4	DeHV-4	*Tursiops truncatus*	Smolarek Benson et al. (2006)	AY952777
Delphinid HV 5	DeHV-5	*Tursiops truncatus*	Smolarek Benson et al. (2006)	AY952776
Delphinid HV 6	DeHV-6	*Grampus griseus*	Smolarek Benson et al. (2006)	DQ288666
Delphinid HV 7	DeHV-7	*Tursiops truncatus*	Smolarek Benson et al. (2006)	AY608707
Delphinid HV 8	DeHV-8	*Tursiops truncatus*	This study	GQ429150
Delphinid HV 9	DeHV-9	*Orcinus orca*	This study	GQ429151
Kogiid HV 1	KoHV-1	*Kogia sima*	Smolarek Benson et al. (2006)	AY949830
Otariid HV 1	OtHV-1	*Zalophus californianus*	Lipscomb et al. (2000)	AF193617
Otariid HV 2	OtHV-2	*Zalophus californianus*	This study	GQ429148
Phocid HV 1	PhHV-1	*Phoca vitulina*	King et al. (1998)	U92269
Phocid HV 2	PhHV-2	*Phoca vitulina*	This study	GQ429152
Phocid HV 3	PhHV-3	*Monachus schauinslandi*	Goldstein et al. (2006a)	DQ093191
Phocid HV 4	PhHV-4	*Mirounga angustirostris*	Goldstein et al. (2006b)	DQ183057
Phocid HV 5	PhHV-5	*Phoca vitulina*	This study	GQ429153
Trichechid HV 1	TrHV-1	*Trichechus manatus*	Wellehan et al. (2008)	DQ238847
Ziphid HV 1	ZiHV-1	*Mesoplodon densirostris*	Saliki et al. (2006)	AY803337

bottlenose dolphin (192 bp) was most closely related to DeHV-3 (AY757301) with an 87% amino acid identity. The translated amino acid sequence from the harbor seal sample (450 bp) was 73% identical to a lymphocryptovirus of a bearded pig (*Sus barbatus*; GenBank accession number AY177148). The orca herpesviral sequence (468 bp) had an amino acid identity of 89% to DeHV-7 (AY608707). BLASTP analysis of the California sea lion sequence (166 bp) showed a 70% identity to *Mustelid herpesvirus 1* (MusHV-1; AF275657). The partial Dpol sequence of PhHV-2 (1308 bp) was also most similar to MusHV-1 with an 82% amino acid identity. Upon further analysis, the California sea lion sequence has an even higher amino acid identity to PhHV-2 (85%) than to MusHV-1. These novel herpesviral Dpol sequences have been submitted to GenBank under the following accession numbers (see Table 1; partial gene sequences, with translation, are available in the online supplementary data.): *Delphinid HV 8* (192 bp from *T. truncatus*; GQ429150), DeHV-9 (468 bp from *O. orca*; GQ429151), *Otariid HV 2* (OtHV-2, 166 bp from *Z. californianus*, GQ429148), *Phocid HV 2* (PhHV-2, 1308 bp from positive control isolate, GQ429152), and PhHV-5 (450 bp from *P. vitulina*; GQ429153).

3.2. Phylogenetic analysis

Bayesian phylogenetic analysis for the larger 45 herpesviral sequence group showed the greatest harmonic mean of estimated marginal likelihoods using the ClustalW2 alignment (for alignment, see online supplementary data). Additionally, the WAG model of amino acid substitution was most probable with a posterior probability of 1.000 (Whelan and Goldman, 2001). Protein maximum likelihood (ProML) analysis showed the most likely tree was also with the ClustalW2 alignment and with the JTT model of amino acid substitution. The Bayesian tree using ClustalW2 alignment is shown in Fig. 1 along with the bootstrap values from the ProML analysis.

The Bayesian phylogenetic analysis using MUSCLE for the 19 alphaherpesviral sequences concluded the WAG model of amino acid substitution was most probable with a posterior probability of 0.930. For the gammaherpesvirus MUSCLE alignment, the Jones model was most probable with a posterior probability of 0.768 followed by the WAG model with a posterior probability of 0.232. ProML analysis showed the most likely tree, for both alignments, was from the JTT model of amino acid substitution and these

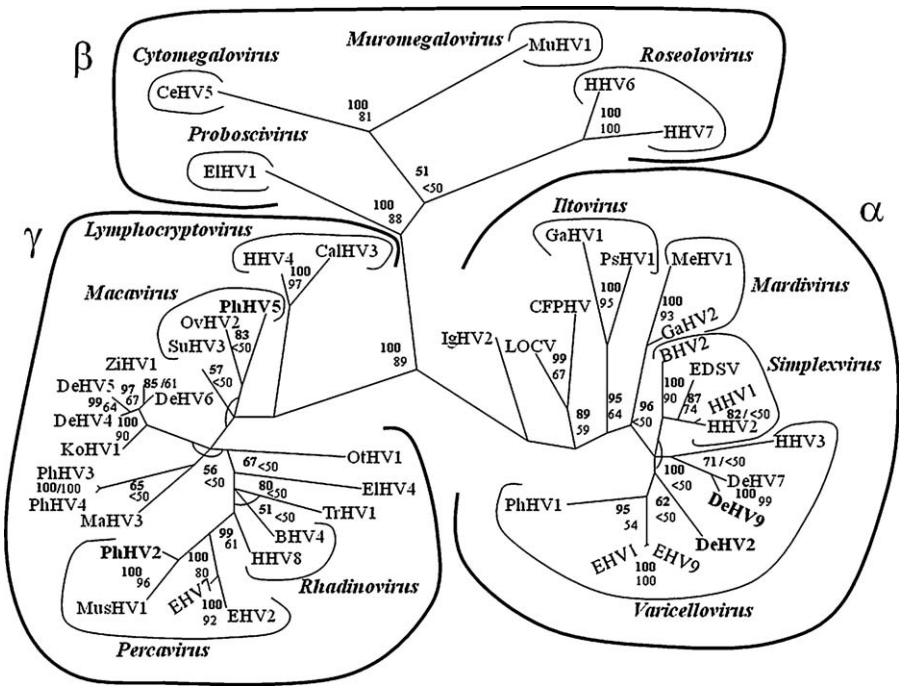

Fig. 1. Bayesian phylogenetic tree of the 45 partial DNA-dependent DNA polymerase predicted amino acid (144–152) herpesviral sequences based on ClustalW2 alignment. Bayesian posterior probabilities of branchings as percentages are in bold and maximum likelihood bootstrap values for branching, based on 100 re-samplings, are given below them. *Iguanid HV 2* (IgHV-2; GenBank accession number AY236869) was used as the outgroup. Herpesvirus genera are delineated by brackets. Areas of multifurcation are marked by arcs. Sequences retrieved from GenBank include (novel sequences from this study are bolded): *Bovine HV 2* (BHV-2; AF181249), BHV-4 (AF318573), *Callitrichine HV 3* (CalHV3; AF319782), *Cercopithecine HV 5* (CeHV5; AY117754), Chelonid fibropapilloma-associated HV (CFPHV; AY644454), *Delphinid HV 2* (DeHV-2; GQ429150), DeHV-4 (AY952777), DeHV-5 (AY952776), DeHV-6 (DQ288666), DeHV-7 (AY608707), DeHV-9 (GQ429151), *Eidolon dupreanum* simplexvirus (EDSV; FJ040878), *Elephantid HV 1* (ElHV-1; AF322977), EHV-4 (DQ238846), *Equid HV 1* (EHV-1; NC_001491), EHV-2 (U20824), EHV-7 (EU165547), EHV-9 (NC_011644), *Gallid HV 1* (GaHV-1; AF168792), GaHV-2 (NC_002229), *Human HV 1* (HHV-1; X14112), HHV-2 (Z86099), HHV-3 (AB059831), HHV-4 (DQ279927), HHV-6 (X83413), HHV-7 (AF037218), HHV-8 (U93872), *Kogiid HV 1* (KoHV-1; AY949830), Loggerhead orocutaneous HV (LOCV; EU004542), *Macropdid HV 3* (MaHV3; EF467663), *Meleagrid HV 2* (MeHV-1; NC_002641), *Murid HV 2* (MuHV2; AY728086), *Mustelid HV 1* (MusHV1; AF275657), *Otariid HV 1* (OtHV-1; AF236050), *Ovine HV 2* (OvHV-2; DQ198083), *Phocid HV 1* (PhHV-1; U92269), PhHV-2 (GQ429152), PhHV-3 (DQ093191), PhHV-4 (DQ183057); PhHV-5 (GQ429153), *Psittacid HV 1* (PsHV-1; NC_005264), *Suid HV 3* (SuHV3; AF478169), *Trichechid HV 1* (TrHV-1; DQ238847), and *Ziphid HV 1* (ZiHV-1; AY949828). IgHV-2 is shown first as the data outgroup.

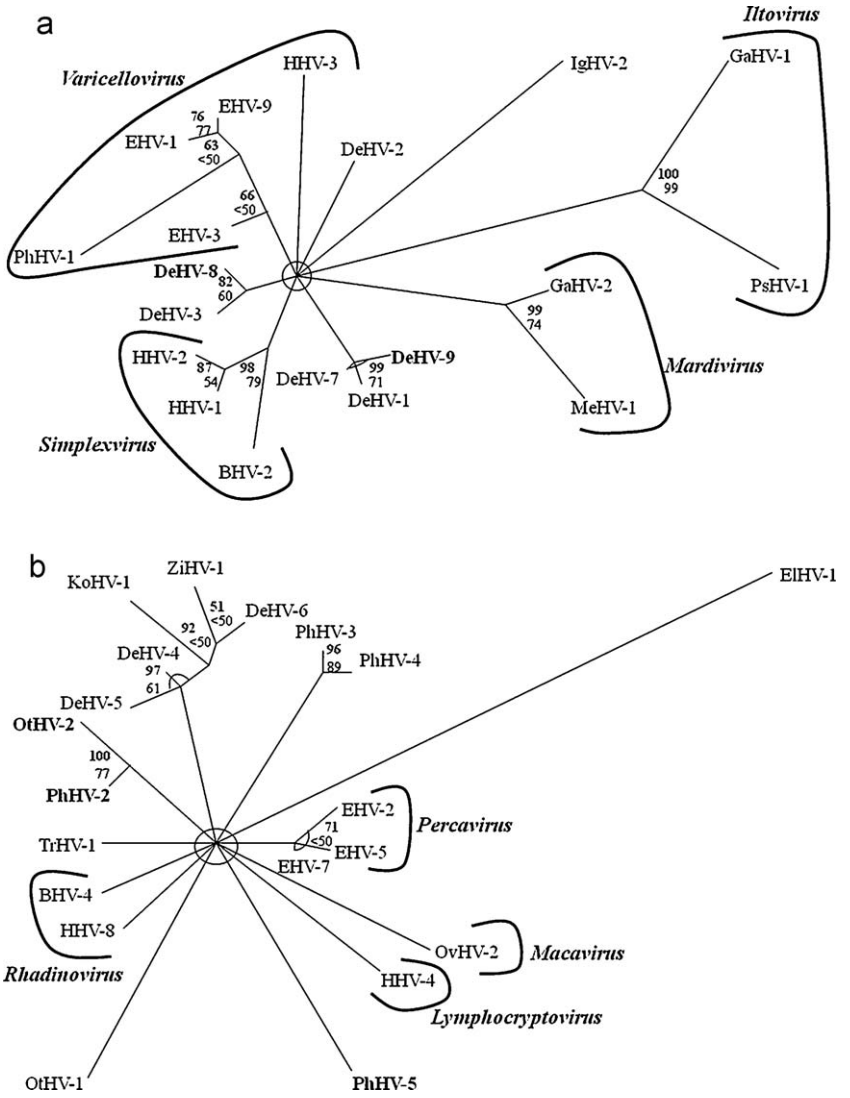

Fig. 2. (a) Bayesian phylogenetic tree of the 19 predicted partial 58–64 amino acid alphaherpesviral DNA-dependent DNA polymerase sequences based on MUSCLE alignment. Bayesian posterior probabilities of branchings as percentages are in bold and ML bootstrap values for branching, based on 100 re-samplings, are given below them. Iguanid HV 2 (GenBank accession number AY236869) was used as the outgroup. Herpesvirus genera are delineated brackets. Areas of multifurcation are marked by arcs. Bottlenose dolphin herpesviruses used in quantitative PCR assays are bolded. Sequences retrieved from GenBank include (novel sequences from this study are bolded): *Bovine HV 2* (BHV-2; AF181249), *Delphinid HV 1* (DeHV-1; AF196646), DeHV-2 (AF245443), DeHV-3 (AY757301), DeHV-7 (AY608707), DeHV-8 (GQ429150), DeHV-9 (GQ429151), *Equid HV 1* (EHV-1; NC_001491), EHV-3 (AF514779), EHV-9 (NC_011644), *Gallid HV 1* (GaHV-1; AF168792), GaHV-2 (NC_002229), *Human HV 1* (HHV-1; X14112), HHV-2 (Z86099), HHV-3 (AB059831), *Iguanid HV 2* (IgHV-2; AY236869), *Meleagrid HV 2* (MeHV-1; NC_002641), *Phocid HV 1* (PhHV-1; U92269), and *Psittacid HV 1* (PsHV-1; NC_005264). (b) Bayesian phylogenetic tree of the 20 predicted partial 55–60 amino acid gammaherpesviral DNA-dependent DNA polymerase sequences based on MUSCLE alignment. Bayesian posterior probabilities of branchings as percentages are in bold and ML bootstrap values for branching, based on 100 re-samplings, are given below them. Elephantid HV 1 (GenBank accession number AF322977) was used as the outgroup. Herpesvirus genera are delineated brackets. Areas of multifurcation are marked by arcs. Sequences retrieved from GenBank include: *Bovine HV 4* (BHV-4; AF318573), *Delphinid HV 4* (DeHV-4; AY952777), DeHV-5 (AY952776), DeHV-6 (DQ288666), *Elephantid HV 1* (ElHV-1; AF322977), *Equid HV 2* (EHV-2; U20824), EHV-5 (AF141886), EHV-7 (EU165547), *Human HV 4* (HHV-4; DQ279927), HHV-8 (U93872), *Kogiid HV 1* (KoHV-1; AY949830), *Otariid HV 1* (OtHV-1; AF236050), OtHV-2 (GQ429148), *Ovine HV 2* (OvHV-2; DQ198083), *Phocid HV 2* (PhHV-2; GQ429152), PhHV-3 (DQ093191), PhHV-4 (DQ183057); PhHV-5 (GQ429153), *Trichechid HV 1* (TrHV-1; DQ238847), and *Ziphid HV 1* (ZiHV-1; AY949828).

parameters were used in the bootstrap analysis. Bayesian phylogenetic trees of the alpha- and gammaherpesvirus MUSCLE alignments with ProML analysis are shown in Fig. 2.

All 19 novel and known marine mammal herpesviruses included in the analyses aligned within the alpha or gamma subfamily of *Herpesviridae* (Figs. 1 and 2). In particular for the pinniped herpesviruses ($n = 7$), all of the sequences aligned with *Gammaherpesvirinae* except PhHV-1 which clustered with varicelloviruses as previously reported (Davison et al., 2009; Harder et al., 1996). PhHV-2 clustered strongly in the genus *Percavirus* (Fig. 1). Furthermore, PhHV-2 clustered with MusHV-1, a representative of the host order Carnivora, while *Equid herpesvirus 2* (EHV-2) and 7 (EHV-7), representatives of Perissodactyla, clustered together yield-

ing a split between viruses of Perissodactyla and Carnivora hosts within *Percavirus*. Incidentally, EHV-7 is currently unassigned to a genus and this analysis showed a strong clustering within *Percavirus* with strong bootstrap support.

For other pinniped herpesviruses, PhHV-3 and 4 clustered strongly together and could not be associated with an established gammaherpesviral genus. Conversely, PhHV-5 clustered with *Macavirus*, a gammaherpesviral genus. However, the ML bootstrap values were weak so the confidence in this cluster is low. Also evident in Fig. 1, OtHV-1 formed a monotypic clade and did not cluster with any of the other gammaherpesvirus clades. While OtHV-2 was only included in the smaller *Gammaherpesvirinae* phylogenetic analysis (since only a smaller Dpol fragment was amplified), it did cluster strongly with PhHV-2 (Fig. 2b).

The delphinid alphaherpesviruses included in the larger analysis (DeHV-2, 7, and 9) all clustered within *Varicellovirus* (Fig. 1). DeHV-2 formed a monotypic clade whereas DeHV-7 and DeHV-9 clustered together in a separate clade. ProML bootstrap values within this genus were weak thus the branching order could not be reliably determined (Fig. 1). The shorter versions of these sequences did not support clustering within *Varicellovirus* as seen in Fig. 2a. Instead, DeHV-1, 7, and 9 clustered together in a monophyletic group and DeHV-2 resided in a monotypic clade without association to established genera. In addition, DeHV-3 and 8 clustered together and were not clearly associated with any other group (Fig. 2a). Lastly, all five cetacean gammaherpesviruses (DeHV-4, 5, 6, KoHV-1, and ZiHV-1) formed a monophyletic clade and could not be associated with an established gammaherpesviral genus (Figs. 1 and 2a).

4. Discussion

The herpesviral sequences in this study are from a single gene, the DNA-dependent DNA polymerase (Dpol). While inclusion of multiple genes is preferable for a robust phylogenetic analysis, it is not feasible for an analysis of marine mammal herpesviruses since few have multiple genes sequenced and many putative marine mammal herpesviruses are amplified from limited clinical samples. As expected for viral polymerases, the Dpol region is highly conserved. This makes it the gene of choice for examining the phylogenetic relationships of distantly related organisms because homologous sites can be more reliably aligned (VanDevanter et al., 1996). Since the sequences can be so similar in this area, each amino acid difference weighs heavily in the analysis. For example, the difference between *Human herpesvirus 1* and *2* is only 8 out of 151 amino acids in this Dpol region which equates to a 94% BLASTP identity. Another benefit of the Dpol region is that most known herpesviruses have published polymerase sequences, thereby yielding a comprehensive library of sequence data for comparison. Incomplete taxa sampling can lead to flawed analyses even when long sequences including multiple genes are employed (Lunter, 2007). This study included a diverse array of herpesviruses to illustrate the evolution of herpesviruses amongst marine mammal host species and in comparison to those of other animals.

The phylogenetic tree topology for the 45 herpesviral sequences is generally consistent with trees generated from other data sets (Smith et al., 2008; Wellehan et al., 2008). This analysis supports the creation of the recent genus *Macavirus* as the Bayesian tree shows an obvious divergence within the gammaherpesviruses. However, the separation of the *Percavirus* genus from *Rhadinovirus* is less apparent. *Rhadinovirus* seems paraphyletic, as with previously reported data sets (Smith et al., 2008; Wellehan et al., 2008). In addition, lack of support for clustering in the smaller data sets, Fig. 2, was not unexpected. However, the separate *Alphaherpesvirinae* and *Gammaherpesvirinae* data sets were included to allow for the addition of four more marine mammal herpesviruses which have sequence lengths less than half of those shown in the *Herpesviridae* phylogenetic analysis (Fig. 1). Fig. 2 is useful for a basic comparison of the shorter marine mammal herpesviral sequences within each subfamily but does not provide data sufficient for further phylogenetic resolution. The diversity of marine mammal herpesviruses is illustrated in the *Herepesviridae* phylogenetic tree by the varied clustering within established genera and the distinct clusters within the gamma subfamily which were not associated with established genera. Furthermore, host fidelity of marine mammal herpesviruses is visible in the segregation of cetacean, pinniped, and sirenian herpesviral clusters.

PhHV-1 remains the sole pinniped herpesvirus in *Alphaherpesvirinae*. However, several delphinid herpesviruses join PhHV-1 in the genus *Varicellovirus*. The strong clustering of DeHV-1 with DeHV-7 and DeHV-9 in the smaller *Alphaherpesvirinae* analysis suggests DeHV-1 is also associated with the *Varicellovirus* genus. Additionally, DeHV-2 clustered in *Varicellovirus* but represents a distinct lineage. Since PhHV-2 clustered strongly with percaviruses in the larger analysis and OtHV-2 clustered strongly with PhHV-2 in the smaller *Gammaherpesvirinae* analysis, OtHV-2 may also reside in this established gammaherpesviral genus. However, more OtHV-2 sequence is needed to confirm this.

PhHV-3 and 4 clustered strongly together and only differed by 4 out of 151 amino acids used in the analysis. Since the herpesviruses are from two different pinniped hosts, Hawaiian monk seal (*Monachus schauinslandi*) and Northern elephant seal (*Mirounga angustirostris*), they were treated as different herpesvirus species in this analysis. More sequence data is necessary for phylogenetic resolution and may indicate that only one isolate should be considered in future analyses. PhHV-5 showed the most basal divergence amongst marine mammal herpesviruses and clustered with representatives of *Macavirus*. However, more representatives and longer sequence are needed to strengthen the confidence values of the group.

Four novel herpesviruses were amplified from marine mammal samples submitted for clinical diagnostics. The viral sequences were compared via BLAST and were clearly herpesviruses. Furthermore, they were sufficiently distinct to be considered novel, as the region sequenced is highly conserved. Bayesian and ML phylogenic analyses support the classification of the isolates as alpha- and gammaherpesviruses. Additional sequence contributions from this work include the partial Dpol region of PhHV-2 and an extension of the partial Dpol sequence for DeHV-2. Further studies assessing phylogenetic relationships amongst

marine mammal herpesviruses should aim to attain more sequence data from more taxa so a better proportion of species are represented. Marine mammal herpesviruses are diverse and host fidelity is evident, as with other mammalian and non-mammalian herpesviruses. Thus, the clinical significance of marine mammal herpesviruses should also be investigated for both endemic and aberrant hosts.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2010.09.035.

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