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Parapoxviruses of seals and sea lions make up a distinct subclade within the genus *Parapoxvirus*

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Abstract

Poxviruses of seals and sea lions have been tentatively identified as both orthopoxviruses and parapoxviruses, but their exact identity remained unconfirmed. Here, poxviral DNA sequences were generated from 39 clinical cases and compared to sequences from earlier poxvirus isolates from seals (*Phocidae*) and sea lions (*Otariidae*). Six genetically distinct poxvirus strains were detected, of which three were previously unrecognized. All detected strains were closely related to the parapoxviruses, confirming their classification as members of the genus *Parapoxvirus*. A phylogenetic analysis showed that pinniped parapoxviruses form a monophyletic group within the genus *Parapoxvirus*. Parapoxviruses from Atlantic pinnipeds were phylogenetically distant from those of Pacific pinnipeds. Parapoxviruses from phocids and otariids that inhabit the same geographical region were also phylogenetically distant, suggesting that parapoxviruses are not commonly transmitted between free-ranging phocids and otariids. However, one strain was detected in two otariid species, suggesting that pinniped parapoxviruses are capable of infecting multiple species within a phylogenetic family.

Keywords: Seal; Sea lion; Pinniped; Poxvirus; Parapoxvirus; Phylogeny; Host range

Introduction

The poxviruses (Family *Poxviridae*) represent a unique family of large, double-stranded DNA viruses that have an entirely cytoplasmic life cycle. The family of poxviruses includes some major pathogens of humans, domestic animals and wildlife (Moss, 2001). The poxvirus family is subdivided into the entomopoxvirus and chordopoxvirus (ChPV) subfamilies, which respectively infect insect and vertebrate hosts (Moss, 2001). The ChPVs are further divided into eight genera (*Avipoxvirus*, *Capripoxvirus*, *Leporipoxvirus*, *Molluscipoxvirus*, *Orthopoxvirus*, *Suipoxvirus*, *Yatapoxvirus* and *Parapoxvirus*).

The recognized members of the genus *Parapoxvirus* currently consist of *Orf virus* (OrfV), *Bovine papular stomatitis virus* (BPSV), *Pseudocowpoxvirus* (PCPV), *Parapoxvirus of red deer in New Zealand* (PVNZ) and *Squirrel parapoxvirus* (SPPV) (Fauquet and Mayo, 2005). The natural hosts of these viruses are wild and domestic ruminants, but OrfV, BPSV and PCPV are known to infect man as well (Mercer et al., 1997). Tentative members of the parapoxvirus group include Auzduk disease virus, Camel contagious ecthyma virus, Chamois contagious ecthyma virus, Sealpox virus and a parapoxvirus

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of California sea lions (Sea Lion Poxvirus-1) (Nollens et al., in press). The seal and sea lion parapoxviruses are considered zoonotic agents as animal handlers have acquired proliferative "sealpox" lesions on their hands after contact with captive, lesion-bearing seals (Clark et al., 2005; Hicks and Worthy, 1987).

In pinnipeds, the group of animals consisting of all seals (Family *Phocidae*), sea lions (Family *Otariidae*) and walruses (Family *Obodenidae*), poxvirus infections manifest clinically as proliferative lesions, 2–3 cm in diameter, on the skin or the mucosal surface of the mouth and nasal passages. Pox lesions are sometimes observed in free-ranging seals and sea lions, but they are especially common as a complication in the treatment of debilitated, stranded pinnipeds in specialized marine mammal rehabilitation centers. The pinniped poxviruses have been tentatively identified as belonging to both the genus *Orthopoxvirus* (Osterhaus et al., 1990) and *Parapoxvirus* (Nettleton et al., 1995; Osterhaus et al., 1994; Simpson et al., 1994), based on morphologic characteristics on positive and negative staining electron microscopy. Molecular investigations of pinniped poxviruses are limited to two accounts, in which two poxvirus strains of, respectively, a European harbor seal (*Phoca vitulina vitulina*) (Becher et al., 2002) and a California sea lion (*Zalophus californianus*) (Nollens et al., in press) were tentatively classified as new members of the genus *Parapoxvirus*.

Features that distinguish parapoxviruses (PPVs) from orthopoxviruses (OPVs) are the elongated virion shape, the crisscross pattern on the particle surface and the relatively high G–C content of the genome (Delhon et al., 2004; Moss, 2001). The PPVs and OPVs have a common evolutionary ancestor and they consequently share features such as size, genome organization and general virion structure (Delhon et al., 2004; Fleming et al., 1993; Mercer et al., 1995). However, only some of the orthopoxvirus and parapoxvirus surface antigens cross-react (Moss, 2001), and the differing nucleic acid content of the genomes sets them genetically apart (Fleming et al., 1993). The phylogenetic classification of the pinniped poxviruses is therefore of importance when developing molecular and serologic diagnostic assays. Additionally, it has been suggested that some parapoxviruses have the capability of causing persistent, recrudescing infections (Iketani et al., 2002). The phylogenetic classification of poxviruses of pinnipeds may therefore also be of importance for future epidemiological and ecological studies.

Extensive molecular phylogenetic studies to resolve identity, taxonomy, evolutionary relationships and host range have not been performed on poxviruses of seals and sea lions. Here, we report on a molecular survey of pinniped poxviruses. The tools for this survey consisted of panparapox and panorthopox PCR protocols that were designed to amplify highly conserved poxvirus genomic regions encoding either the p37K envelope phospholipase of orthopoxviruses and its equivalent in parapoxviruses or the hemagglutinin protein of orthopoxviruses. The aim of this study was to further clarify the phylogenetic identity of the pinniped poxviruses and at evaluating the role host range and geographic isolation may have on the evolutionary divergence of these viruses.

Results

Animals and samples

In total, PCR amplifications were performed on 110 pox samples from 64 seals and sea lions (Table 1) using each of the following four primer sets: Para1/Para2, Ortho1/Ortho2, EACP1/EACP2 and NACP1/NACP2. The universal panparapox primer pair Para1/Para2, amplifies part of the genomic region (B2L gene) encoding the putative virion envelope antigen (p42K) of parapoxviruses (Inoshima et al., 2000; Sullivan et al., 1994). The orthopoxvirus-specific primer pair Ortho1/Ortho2 was designed to amplify a 546 bp product of the genomic region (F13L) encoding the EEV phospholipase (p37K) of orthopoxviruses, the orthopoxvirus orthologue of the p42K of parapoxviruses. The primer pairs EACP1/EACP2 and NACP1/NACP2 are panorthopox primers, designed to amplify the genomic region encoding the hemagglutinin protein (HA) of, respectively, Old World and New World orthopoxviruses (Ropp et al., 1995).

Poxviral DNA was detected in 55 (50%) samples from 39 animals (Table 1). Twenty-two (63%) biopsy or necropsy samples, three (60%) paraffinized or formalinized tissues and 30 (43%) pox lesion swabs derived from 30 California sea lions, one grey seal, five West Atlantic harbor seals and three Pacific harbor seals (*Phoca vitulina richardii*) were found to contain virus (Table 1). All 55 viral-derived amplicons were obtained using the Para1/Para2 panparapox primers. No poxviral DNA was detected using any of the three primer sets specific for OPV (Ortho1/Ortho2, EACP1/EACP2 and NACP1/NACP2) (Table 1).

Viral sequence analyses

The multiple nucleotide sequence alignment showed that six distinct virus strains were detected in the 55 positive samples

Table 1
Number of animals of each host species from which samples were received (*N*), number of animals in which a poxvirus was detected (Pos) and poxvirus strain detected using the Ortho, EACP, NACP and Para primer pairs

Host species	*N*	Pos	Ortho	EACP	NACP	Para					
						SLPV-1	SLPV-2	SLPV-3	GSPV-1	HSPV-1	HSPV-2
California sea lion	53	30	–	–	–	16	12	2	–	–	–
Grey seal	1	1	–	–	–	–	–	–	1	–	–
Harbor seal (West Atlantic)	5	5	–	–	–	–	–	–	–	5	
Harbor seal (Pacific)	5	3	–	–	–	–	–	–	–	–	3

(Fig. 1). One virus strain was only found in grey seals (Grey Seal Poxvirus-1; GSPV-1) (Tables 1 and 2, Fig. 1). Similarly, two virus strains were only found in harbor seals from the Western Atlantic (Harbor Seal Poxvirus-1; HSPV-1) or the Pacific (Harbor Seal Poxvirus-2; HSPV-2). Three virus strains were detected in California sea lions (Sea Lion Poxvirus-1, 2, 3; SLPV-1, 2, 3). All generated sequences were equal in length (554 bp, excluding primers). A total of 119 (22%) nucleotide positions were variable sites, defined as containing at least two types of nucleotides, but no hypervariable hot spot regions could be identified (Fig. 1).

The pairwise nucleotide and amino acid sequence comparisons between the all generated viral sequences and sequences from available poxvirus sequences from seals and sea lions showed that sequence HSPV-1 amplified from West Atlantic harbor seals was identical to sequence P.vitulina-V465 previously amplified from West Atlantic harbor seals (Tables 2 and 3). Additionally, SLPV-3, here amplified from California sea lions, was identical to sequence E.jubatus-V841 that was previously amplified from Steller sea lions (Tables 2 and 3). P. vitulina-V465 and E.jubatus-V841 are therefore represented by, respectively, HSPV-1 and SLPV-3 in all subsequent phylogenetic analyses. No virus strains were detected in multiple host species of the phocid family.

The mean nucleotide and amino acid *P* distances between all pinniped poxviruses was 0.079 and 0.049, respectively. Of all pinniped poxviruses, P.vitulina-NSea and P.vitulina-V465 were most closely related to each other (nucleotide $P = 0.002$) and SLPV-2 was most divergent from all other pinniped poxviruses (Table 3). Pinniped poxviruses from the Atlantic ocean appeared least genetically diverse (nucleotide $P_{\text{within Atlantic}} = 0.018 \pm 0.004$). Pinniped poxviruses from the Pacific ocean were even more divergent from each other (nucleotide $P_{\text{within Pacific}} = 0.096 \pm 0.009$) than from those from the Atlantic (nucleotide $P_{\text{between Atlantic – Pacific}} = 0.084 \pm 0.008$). Phocid poxviruses of the Pacific were more closely related to each other (nucleotide $P_{\text{within Pacific phocid}} = 0.051 \pm 0.008$) than to otariid poxviruses of the Pacific (nucleotide $P_{\text{between Pacific phocid – Pacific otariid}} = 0.093 \pm 0.009$).

The topology of a phylogenetic tree consisting of homologous sequences derived from all pinniped poxviruses in reference to all PPVs and the protypal members of all other ChPV genera showed that all six newly detected strains were most closely related to the known PPVs (Fig. 2). This confirms their classification as members of the genus *Parapoxvirus*. The tree topology further showed that pinniped PPVs form a distinct, monophyletic clade within the genus *Parapoxvirus* with a node certainty of 96%. Of the known pinniped PPVs, SLPV-2 is most closely related to the root or ancestral pinniped PPV.

The phylogenetic analysis of the nine pinniped PPVs alone demonstrated that PPVs from Atlantic pinnipeds (P.vitulina-NSea, HSPV-1, GSPV-1) were phylogenetically distant from most Pacific pinniped PPVs (P.largha, HSPV-2, E.jubV1346, SLPV-1, SLPV-2, SLPV-3) (Table 2, Fig. 3). Similarly, PPVs from Pacific phocid (P.largha, HSPV-2) and otariid (E. jubV1346, SLPV-1, SLPV-2, SLPV-3) host species within the same geographical region were also phylogenetically distant from each other (Fig. 4).

Discussion

All pinniped poxviruses detected in this study were most closely related to the known PPVs. This confirms the earlier tentative classification of the poxviruses of seals and sea lions as members of the genus *Parapoxvirus*. Earlier phylogenetic classifications of the poxviruses of pinnipeds were usually solely based on the characteristics of surface tubules of purified virions on negative staining EM. However, it has been suggested that these surface characteristics are preparation artifacts induced by osmotic stress imposed during negative staining and that in reality poxvirions have a smooth surface (Dubochet et al., 1994). While other evidence has since been presented suggesting that the appearance of the surface tubules is not artifactual (Heuser, 2005, Malkin et al., 2003, Wilton et al., 1995), sequencing of viral genes is the current method of choice for identifying poxviruses. In only one report were data on pinniped pox virion surface morphology supplemented with viral genomic sequence to unequivocally assign a poxvirus of pinnipeds to the genus *Parapoxvirus* (Nollens et al., in press). No orthopoxviruses were detected in any of the samples. It is possible that the genomic sequences of orthopoxviruses would be too divergent to be amplified by the degenerate pan-orthopox primer pairs used in this study; however, because of the absence of appropriate controls, this cannot be verified.

It appears that the natural hosts of PPVs consist solely of ruminants and pinnipeds. Interestingly, ruminants are the closest extant terrestrial relatives of the cetacean group of marine mammals consisting of all whales, dolphins and porpoises. However, the poxviruses associated with whales and dolphins species are (tentatively) classified as orthopoxviruses (Van Bressem et al., 1993). In contrast, the closest terrestrial relatives of pinnipeds are the members of the suborder Ursidae and the other members of the Order Carnivora, but the only reported cases of poxvirus infections in carnivores are accidental infections with the orthopoxvirus cowpox virus (Meyer et al., 1999, Pelkonen et al., 2003, Smith et al., 1999).

The pinniped PPVs form a distinct, monophyletic clade within the genus *Parapoxvirus* (node certainty of 96%), thus setting the PPV of pinnipeds genetically apart from all other PPV. The International Committee on the Taxonomy of Viruses defines a virus species as a polythetic class of viruses that constitute a replicating lineage and occupy a particular ecological niche (Pringle, 1991). Because of their genetic relatedness, their exclusive occurrence in the marine environment and their distinctive natural host range, the polythetic class of pinniped parapoxviruses could be recognized as a separate species within the genus *Parapoxvirus*.

Phylogenetic analyses based on single genes may give rise to inaccurate tree topologies. In order to gather further support for the branching order observed in this study, we compared the results of the topology of phylogenetic tree generated in this

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GSPV-1    TAC CGG CGG CTC GCT AGC CAC TAT TAA AAA CCT GGG CGT GTA CTC CAC
SLPV-1    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HSPV-1    ... ... ... ... ... ... ... C.. ... ... .T. ... ... ... ... ...
HSPV-2    ... ... ... ... ... ... T.. C.. ... ... .T. ... ... ... ... ...
SLPV-3    ... T.. ... ... ... G.. ... C.. ... ... ... ... ... ... ... ...
SLPV-2    C.. ... ... ... ... G.. T.. C.. C.. G.. ... ... A.. ... ... G..

GSPV-1    CAA CAA GCA CTT GGC TGT CGA CCT CAT GAA CAG GTA CAA CAC CTT TAG
SLPV-1    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HSPV-1    ... ... ... ... ... C.. T.. ... ... ... ... ... ... ... ... ...
HSPV-2    ... ... ... ... ... ... T.. ... T.. ... .C. ... ... ... T.. ...
SLPV-3    ... ... ... ... ... C.. ... ... ... ... .C. ... ... ... ... ...
SLPV-2    ... T.. ..G .C. ... CT. ... ... ... ... ... ... ... ... ... CG.

GSPV-1    CTC CAT GGT CGT GGA CCC CAA GCA GCC GTT TAC GCG CTT CTG CTG CGC
SLPV-1    ... ... ... T.. ... ... ... ... ... ... ... ... ... ... ... ...
HSPV-1    ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ...
HSPV-2    ... ... ... T.. A.. ... ... ... ... ... ... ... A.. ... ... ...
SLPV-3    ... ... ... T.. ... ... ... ... ... ... C.. ... .A. ... ... ...
SLPV-2    G.. A.. ... ... A.. ... ... ... ... ... CG. A.. AC. ... T.. ...

GSPV-1    CAT GAT AAC GCC CAC AGC CAC GGA CTT CCA CAT GAA CCA CTC TGG CGG
SLPV-1    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HSPV-1    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HSPV-2    ... ... C.. A.. ... G.. ... ... ... ... ... ... ... ... ... ...
SLPV-3    ... ... T.. ... ... G.. ... ... ... T.. ... .C. ... ... ... G..
SLPV-2    ... C.. C.. T.. G.. G.. ... ... ... ... T.. ... T.. ... A.. ...

GSPV-1    CGG CGT TTT TTT CTC AGA CTC ACC AGA GCG CTT CTT GGG CTT CTA CCG
SLPV-1    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HSPV-1    ... ... ... ... ... G.. ... ... ... ... ... ... ... ... ... ...
HSPV-2    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
SLPV-3    ... ... ... C.. T.. ... ... ... ... ... ... ... ... ... ... ...
SLPV-2    T.. ... ... ... ... G.. ... C.. ... ... ... .C. ... A.. ... ...

GSPV-1    CAC GCT CGA CGA AGA CCT GGT GCT GCA CCG CAT CGA CGC TGC AGA AAA
SLPV-1    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HSPV-1    ... ... ... ... ... ... ... ... ... ... ... ... T.. ... ... GA. ...
HSPV-2    ... ... ... ... ... .T. ... C.. ... ... ... ... T.. ... G.. ...
SLPV-3    ... ... ... ... ... ... ... ... ... ... ... ... ... ... GA. ...
SLPV-2    ... ... ... ... ... ... ... ... .T. ... ... ... ... TT. C.. G.. ...

GSPV-1    CAG CAT TGA CCT CTC TCT GCT GTC TAT GGT GCC AGT GGT GCG CTC TGG
SLPV-1    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HSPV-1    ... ... ... ... ... G.. A.. ... ... ... ... ... ... ... ... ...
HSPV-2    ... ... ... ... ... G.. ... ... ... .C. ... ... ... ... ... ...
SLPV-3    ... ... C.. ... ... G.. ... ... C.. ... ... G.. ... ... ... C..
SLPV-2    ... ... C.. T.. A.. A.. ... ... C.. ... ... G.. ... ... ... C..

GSPV-1    CAG CGA GGT GTA CTA CTG GCC GCT GAT CAT GGA CGC GTT GCT GCG CGC
SLPV-1    ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ...
HSPV-1    .G. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HSPV-2    ... ... A.. .C. ... ... ... ... ... ... ... ... ... ... ... ...
SLPV-3    .G. ... ... ... ... ... ... ... ... ... ... ... ... ... ... A..
SLPV-2    ... ..C C.. TC. ... ... ... A.. ... A.. ... ... .C. ... ... ...

GSPV-1    TGC TAT CAA CCG CAG CGT GCG CGT GCG CAT CAT CGT CAG CCA GTG GCG
SLPV-1    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HSPV-1    ... ... ... ... ... ... ... ... ... ... ... T.. ... ... ... ...
HSPV-2    C.. ... T.. ... ... ... ... ... ... ... ... .A. ... ... ... ...
SLPV-3    C.. .G. .G. ... ... ... ... T.. ... ... ... .A. ... ... ... ...
SLPV-2    C.. G.. AG. ... ... ... A.. ... ... T.. ... .A. ... ... ... ...

GSPV-1    TAA CGC GGA TCC ACT GTC CGT GGC TGC AGT TCG TGC GCT GGA CAA CTT
SLPV-1    ... ... ... ... ... ... T.. ... ... ... ... ... ... ... ... ...
HSPV-1    ... ... ... C.. ... ... T.. ... ... ... ... ... ... ... ... ...
HSPV-2    ... ... ... ... G.. ... T.. A.. ... ... ... ... ... ... ... ...
SLPV-3    ... ... ... ... G.. ... ... ... ... ... ... C.. ... ... ... ...
SLPV-2    C.. ... ... ... G.. ... ... ... A.. G.. G.. C.. A.. ... TG. T..

GSPV-1    TGG AGT GGG GCA TAT TGA CAT TAC TGC GCG CTG GTT CGC AAT TCC AGG
SLPV-1    ... ... ... ... ... C.. ... C.. ... ... ... ... ... ... ... ...
HSPV-1    ... ... ... ... .G. C.. ... C.. ... ... ... ... ... ... ... ...
HSPV-2    ... G.. ... ... CG. ... .G. ... ... ... ... ... ... .G. G.. ...
SLPV-3    ... ... ... ... ... C.. ... C.. C.. ... ... ... ... G.. A.. ...
SLPV-2    C.. ... ... ... C.. C.. ... C.. A.. ... ... ... T.. GG. A.. G..

GSPV-1    CCG CGA CGA CGC ATC CAA CAA CAC TA 554
SLPV-1    ... ... ... ... G.. ... ... ... .. 554
HSPV-1    ... ... ... ... ... ... ... ... .. 554
HSPV-2    ... ... ... ... GG. ... ... ... .. 554
SLPV-3    ... ... ... ... G.. ... ... ... .. 554
SLPV-2    A.. T.. ... ... C.. T.. ... ... C. 554
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Fig. 1. Multiple alignment of the partial genomic sequences encoding the putative virion EEV protein (p42K) of the pinniped parapoxvirus strains detected in this study. This sequence alignment was generated using ClustalW and edited using MEGA3.1.

Table 2
Host species, tentative designations and GenBank accession numbers for genomic sequences of pinniped poxvirus strains analyzed in this study

Pinniped host			Poxvirus	
Family	(Sub)species	Common name	Designation	Accession number
Otariidae	*Eumatopias jubatus*	Steller sea lion	E.jubatus-V1346	AY952946.1
			E.jubatus-V841	AY952940.1
	Zalophus californianus californianus	California sea lion	SLPV-1	DQ163058 [a]
			SLPV-2	DQ273137 [a]
			SLPV-3	DQ273138 [a]
Phocidae	*Phoca largha*	Spotted seal	P.largha	DQ073805.1
	Phoca vitulina vitulina	Harbor seal (East Atlantic)	P.vitulina-NSea	AF414182.1
	Phoca vitulina concolor	Harbor seal (West Atlantic)	P.vitulina-V465	AY952937.1
	Phoca vitulina concolor	Harbor seal (West Atlantic)	HSPV-1	DQ273135 [a]
	Phoca vitulina richardii	Harbor seal (Pacific)	HSPV-2	DQ273136 [a]
	Halichoerus grypus	Grey seal	GSPV-1	DQ273134 [a]

[a] Genomic sequences generated as part of this study.

study to a phylogenetic analysis of all ChPV, except the PPV, based on the sequence of 17 conserved poxvirus proteins (Gubser et al., 2004). Both phylogenetic analyses outline the same main groupings of ChPVs in the same branching order. Similarly, the topology of the phylogenetic tree generated here was near identical to that of a phylogenetic tree of PPVs and selected ChPVs that was generated using concatenated gene sequences encoding core and envelope proteins (Tikkanen et al., 2004).

Parapoxviruses from Atlantic pinnipeds were phylogenetically distant from those of Pacific pinnipeds, although SLPV-1 and GSPV-1 do appear to share a more recent evolutionary ancestor (Fig. 3). The Atlantic and Pacific ocean basins are separated by the North and South American continent, but indirect contact between Atlantic and Pacific pinnipeds and occasional spill-over of their pathogens could occur via poxvirus infections of Arctic ice seals (ringed seals (*Phoca hispida*), bearded seals (*Erignathus barbatus*), ribbon seals (*Phoca fasciata*), hooded seals (*Cystophora cristata*), harp seals (*Phoca groenlandica*)). While it is possible that both SLPV-1 and GSPV-1 temporarily accumulated parallel nucleic acid changes, it appears much more likely that their ancestral virus was introduced into both ocean basins. Unfortunately, we were unable to obtain pox lesion samples from the Arctic ice seal species during this study and consequently have insufficient data on poxviruses of ice seals to help verify this hypothesis. Parapoxviruses from phocids and otariids that inhabit the same geographical region were also phylogenetically distant. This indicates that parapoxviruses are not commonly transmitted between free-ranging phocids and otariids (Fig. 4), while this may be biologically possible. Here, the effect of biological isolation (host range) and geographic isolation could be confounded, since no otariid species inhabit the North Atlantic ocean. To correct for this potential confounding effect, only the genetic relatedness of Pacific phocid and Pacific otariid PPV strains was evaluated (Fig. 4). The calculated genetic distances (*P* distance) confirmed that the pinniped PPVs from the Pacific basin were genetically divergent from those from the Atlantic. Additionally, phocid PPVs of the Pacific were more closely related to each other than to the Pacific otariid PPVs. The

Table 3
Genetic diversity of parapoxviruses of North American pinnipeds determined using average *P* distances calculated with MEGA (version 3.1)

	Otariidae					*Phocidae*					
	E.jubatus-V1346	E.jubatus-V841	SLPV-1	SLPV-2	SLPV-3	P.largha	P.vitulina-NSea	P.vitulina-V465	HSPV-1	HSPV-2	GSPV-1
Otariidae											
E.jubatus-V1346	–	0.038	0.038	0.082	0.038	0.038	0.043	0.038	0.038	0.038	0.033
E.jubatus-V841	0.056	–	0.043	0.082	**0.000**	0.065	0.038	0.033	0.033	0.065	0.038
SLPV-1	0.061	0.063	–	0.076	0.043	0.043	0.027	0.022	0.022	0.043	0.005
SLPV-2	0.153	0.148	0.161	–	0.082	0.076	0.092	0.087	0.087	0.076	0.071
SLPV-3	0.056	**0.000**	0.063	0.148	–	0.065	0.038	0.033	0.033	0.065	0.038
Phocidae											
P.largha	0.045	0.090	0.067	0.159	0.090	–	0.049	0.043	0.043	0.011	0.038
P.vitulina-NSea	0.074	0.072	0.034	0.166	0.072	0.076	–	0.005	0.005	0.049	0.016
P.vitulina-V465	0.072	0.070	0.032	0.164	0.070	0.074	0.002	–	**0.000**	0.043	0.016
HSPV-1	0.072	0.070	0.032	0.164	0.070	0.074	0.002	**0.000**	–	0.043	0.016
HSPV-2	0.045	0.090	0.063	0.161	0.090	0.014	0.069	0.067	0.067	–	0.038
GSPV-1	0.065	0.067	0.011	0.159	0.067	0.067	0.034	0.032	0.032	0.063	–

The *P* distance calculated from the nucleotide sequence data is shown below the diagonal and the value calculated from predicted amino acid sequences (AA) is shown above the diagonal. This *P* distance is the proportion of sites at which the two sequences to be compared is different. Identical parapoxviruses ($P = 0.000$) are indicated using bold font.

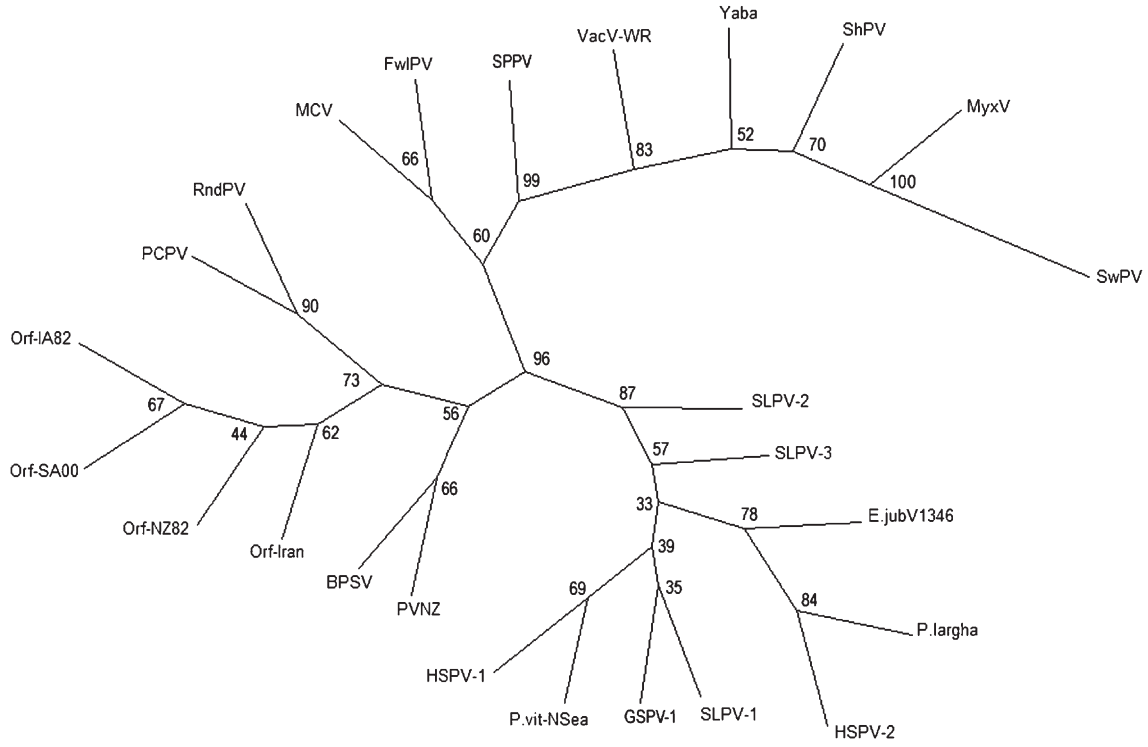

Fig. 2. Phylogram depicting the relationship of the pinniped poxviruses to the published members of the genus *Parapox* [Orf virus strains NZ2, SA00, IA82 and Iran (Orf-NZ2, UO6671.1; Orf-SA00, AY386264.1; Orf-IA82, AY386263; Orf-Iran, AY958203.1), bovine papular stomatitis virus strain BV-AR02 (BPSV, AY386265.1), pseudocowpox virus (PCPV, AY424972.1), red deer parapoxvirus (PVNZ, AB044794.1) and Reindeer poxvirus (RndPV, AY453652)]; previous pinniped parapoxvirus isolates [North Sea harbor seal parapoxvirus (P.vit-NSea), the spotted seal parapoxvirus (P.largha) and a Steller sea lion parapoxvirus (E.jubV1346)] and the protypal members of all other *Chordopox* genera [vaccinia virus strain WR (VacV-WR, AY243312.1), squirrel poxvirus (SPPV, AY340985.1), Swinepox virus isolate 17077-99 (SwPV, AF410153.1), Sheeppox virus strain A (ShPV, AY077833.1), Molluscum contagiosum virus subtype 1 (MCV, U60315.1), Myxoma virus strain Lausanne (MyxV, AF170726.2), Yaba monkey tumor virus (Yaba, AY386371.1) and Fowlpox virus (FwlPV, AF198100.1)]. This phylogenetic tree is based on the 184 translated amino acids of the putative sea lion p42K fragment and homologous regions from the other poxviruses, and was generated according to the maximum-likelihood method using the PHYLIP 3.65. Branch lengths are proportional to genetic distances. Numbers indicate the robustness of each node, defined as the percentage of 100 bootstrap replicates that support each interior branch.

genetic distances between these various groups is in agreement with the topology of the phylogenetic trees (Figs. 3 and 4) and the hypothesis that both geographic isolation and host range are driving factors for the evolutionary divergence of the pinniped PPVs.

Here we also present the first evidence that PPVs of pinnipeds are capable of infecting multiple pinniped host species. We detected one pinniped PPV strain (SLPV-3) in pox lesions obtained from two California sea lions that had previously been detected by a different laboratory in pox lesions from a free-ranging Steller sea lion (E.jubatus-V841; Tables 2 and 3). California sea lions and Steller sea lions are distinct species, but both are members of the Family Otariidae. Steller sea lions inhabit the North Pacific coasts but occur as far south as the Washington coast. The geographic range of California sea lions is centered around the California coast but extends northward into Canada. Where the habitat of these two otariid species overlaps, direct and indirect inter-specific contact is possible. Regardless of the mode of transmission, this finding is in agreement with the abovementioned hypothesis that pinniped PPVs are capable of infecting multiple pinniped host species within one phylogenetic family.

Materials and methods

Animals and samples

A total of 110 clinical samples, from 53 California sea lions (*Z. californianus*), 10 harbor seals (*P. vitulina richardii*; $n = 5$; *P.v.concolor*, $n = 5$) and 1 grey seal (*Halichoerus grypus*) showing clinical signs of a cutaneous poxvirus infection were collected between 15 August 2002 and 14 June 2005 for PCR analysis (Tables 1 and 2). The samples consisted of 35 biopsy or necropsy samples, 5 paraffinized or formalinized tissues and 70 pox lesion swabs. All received samples were collected from stranded seals and sea lions that were admitted to specialized marine mammal rehabilitation centers [The California Marine Mammal Center (CA), Marine Mammal Stranding Center (NJ), Animal Health Center (British Columbia), Mystic Aquarium (CT), Sea World of Orlando (FL), Sea World of San Diego (CA) or the Virginia Marine Science Museum (VA)]. DNA was extracted from all pox lesion scabs and swabs, and from a 25 mg fragment of all tissue samples using a DNeasy Tissue Kit (Qiagen, Valencia, CA), following the manufacturer's guidelines.

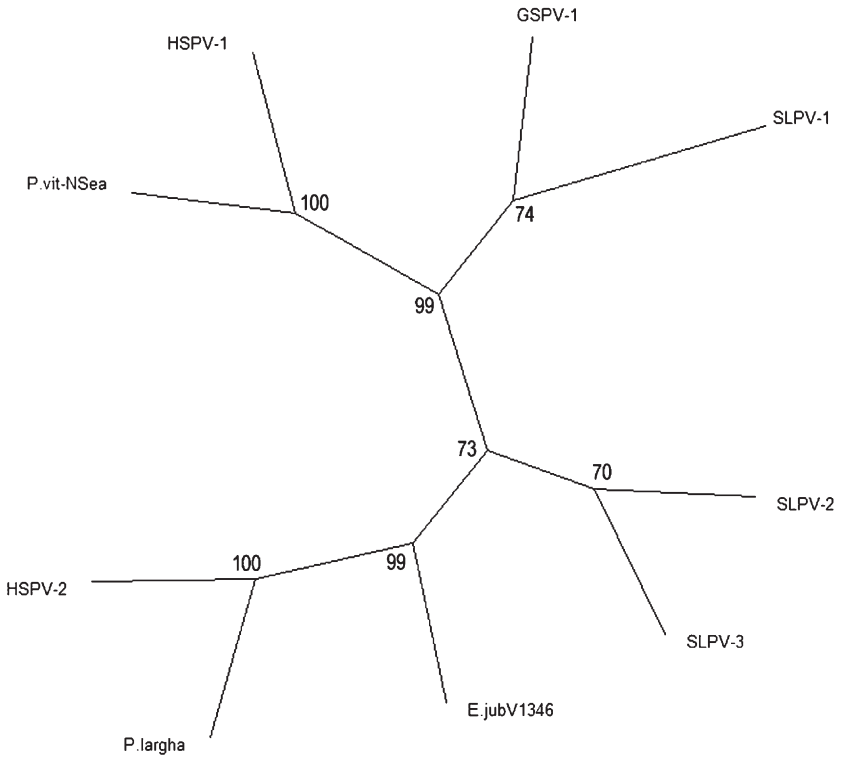

Fig. 3. Phylogram depicting the relationship between the pinniped poxviruses of the Atlantic (P.vit-NSea, HSPV-1 and GSPV-1) and the Pacific Ocean (SLPV-1, SLPV-2, SLPV-3, HSPV-2, E.jubV1346, P.largha). This phylogenetic tree is based on the 184 translated amino acids of the putative sea lion p42K fragment and homologous regions from the other poxviruses, and was generated according to the maximum-likelihood method using the PHYLIP 3.65. Branch lengths are proportional to genetic distances. Numbers indicate the robustness of each node, defined as the percentage of 100 bootstrap replicates that support each interior branch.

Polymerase chain reactions (PCR)

PCR amplifications were performed on each sample and using each of the following four primer sets: Para1/Para2, Ortho1/Ortho2, EACP1/EACP2 and NACP1/NACP2. A universal panparapox primer pair (Para1/Para2, GenBank accession number U06671: sense 5′–3′: GTCGTCCACGATGAGCAGCT and antisense 5′–3′: TACGTGGGAAGCGCCTCGCT) was used to amplify part of the genomic region (B2L gene) encoding the putative virion envelope antigen (p42K) of parapoxviruses (Inoshima et al., 2000, Sullivan et al., 1994). The orthopoxvirus-specific primer pair Ortho1/Ortho2 (sense 5′-CTGGTAGAAACACTACCAGAAAATATGGA-3′ and antisense 5′-TCTTAATATGATACGCAGTGCTAACTGG-3′) was designed to amplify a 546 bp product of the genomic region (F13L) encoding the EEV phospholipase (p37K) of orthopoxviruses, the orthopoxvirus orthologue of the p42K of parapoxviruses. The primer sets EACP1/EACP2 and NACP1/NACP2 are panorthopox primers, designed to amplify the genomic region encoding the hemagglutinin protein (HA) of, respectively, Old World and New World orthopoxviruses (Ropp et al., 1995). An aliquot of 15 μl of the extracted DNA of each sample was used in 100 μl amplification reactions containing 10× PCR buffer, 1 mM $MgCl_2$, 200 μM of each dNTP, 1 μM of each primer and two units of *Taq* polymerase (Roche Diagnostics Corporation, Indianapolis, IN). For each reaction, three wells containing DNA from wild type Orf virus (ORFV), vaccinia virus (Vac-WR) and no DNA were included as positive and negative controls. All four PCR protocols were optimized for yield and specificity using either VacV-WR or ORFV DNA as substrate. Final amplification conditions were: incubation at 94 °C for 2.5 min, and 30 cycles of denaturation at 94 °C for 30 s, 1 min annealing (57 °C for Para1/Para2, 53 °C for Ortho1/Ortho2, 53 °C for EACP1/EACP2 and 54 °C for NACP1/NACP2), and extension at 72 °C for 40 s, followed by incubation at 72 °C for 10 min. All amplicons of controls and unknown samples were purified using a High Pure PCR Product Purification Kit (Roche Diagnostics Corporation, Indianapolis, IN) following the manufacturers instructions, and submitted to the Sequencing Core of the UF-ICBR for nucleotide sequencing. Each nucleotide position was sequenced at least three times in each direction and all primer sequences were excluded from the subsequent analyses. Vector NTI software (Informax, Frederick, MD) was used to analyze and construct contiguous nucleotide sequences.

Viral sequence analyses

A multiple sequence alignment of the generated nucleotide sequences was generated using ClustalW software. The aligned sequences were subsequently edited and analyzed using the MEGA software package version 3.1 (Kumar et al., 2004) (Fig. 1). The derived amino acid sequences of each of the generated sequences were obtained using Vector NTI. Pairwise nucleotide and amino acid comparisons between the all generated viral sequences and all available poxvirus

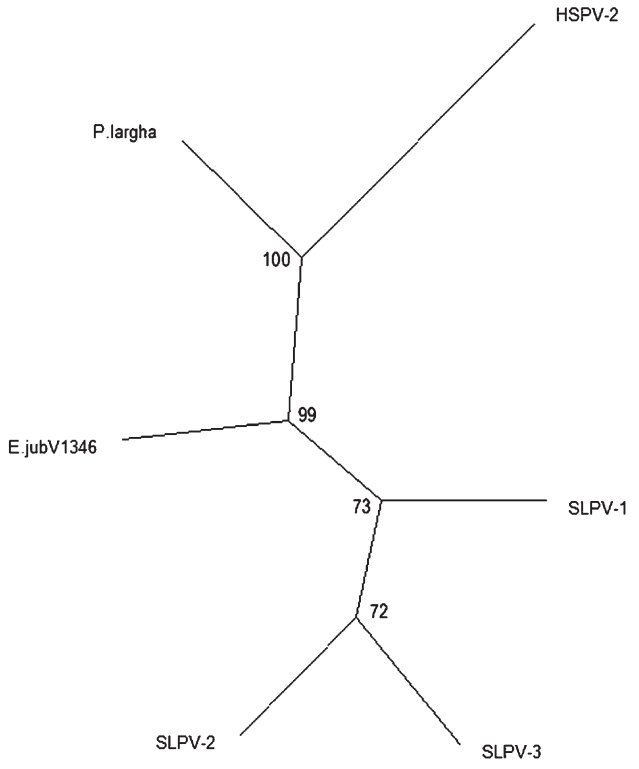

Fig. 4. Phylogram depicting the relationship between the poxviruses of Pacific phocids (HSPV-2, P.largha) and otariids (SLPV-1, SLPV-2, SLPV-3, E.jubV1346). This phylogenetic tree is based on the 184 translated amino acids of the putative sea lion p42K fragment and homologous regions from the other poxviruses, and was generated according to the maximum-likelihood method using the PHYLIP 3.65. Branch lengths are proportional to genetic distances. Numbers indicate the robustness of each node, defined as the percentage of 100 bootstrap replicates that support each interior branch.

sequences from seals and sea lions were conducted using MEGA 3.1 (Table 3). Available pinniped poxvirus sequences included Sea Lion Poxvirus-1 (DQ163058, SLPV-1), two Steller sea lion parapoxvirus sequences (AY952940, E.jubatus-V841; AY952946, E.jubatus-V1346), spotted seal (DQ073805.1, P.largha) and two harbor seal parapoxvirus sequences (AF414182.1, P.vitulina-NSea; AY952937.1 P. vitulina-V465) (Table 2). The nucleotide sequences of two poxviruses of a European grey seal and a Weddell seal (*Leptonychotes weddellii*) (respective GenBank accession numbers AJ622901.1 and AJ622900.2) contain stop codons although the panparapox primers Para1/Para2 target the open reading frame of the orf virus B2L gene equivalent. As a result, these two sequences were excluded from this analysis. For each nucleotide and amino acid pair, the *P* distance, defined as the proportion of sites at which the two sequences to be compared is different, was calculated (Table 3). Additionally, the *P* distances within and between each of the following groups were calculated using Mega3.1: Atlantic poxviruses, Pacific poxviruses, Pacific phocid poxviruses and Pacific otariid poxviruses. No otariid species inhabit the Atlantic ocean. *P* distance within a group was defined as the arithmetic average of all computed pairwise comparisons within that group. *P* distance between groups was defined as arithmetic average of all computed inter-group pairwise comparisons (Kumar et al., 2004).

To clarify the genetic relationships between pinniped poxviruses, published PPVs and other members of the ChPVs, phylogenetic trees were constructed from alignments of the translated envelope protein sequences (Fig. 2). Sequences included in the analysis were Orf virus strains NZ2, SA00, IA82 and Iran (Orf-NZ2, UO6671.1; Orf-SA00, AY386264.1; Orf-IA82, AY386263; Orf-Iran, AY958203.1), bovine papular stomatitis virus strain BV-AR02 (BPSV, AY386265.1), pseudocowpox virus (PCPV, AY424972.1), red deer parapoxvirus (PVNZ, AB044794.1) and Reindeer poxvirus (RndPV, AY453652). Previous pinniped parapoxvirus isolates included the North Sea harbor seal parapoxvirus (P.vit-NSea), the spotted seal parapoxvirus (P.largha) and a parapoxvirus detected in Steller sea lions (E.jubV1346). Also included were the protypal viruses of all other *Chordopox* genera: genus *Orthopox*: vaccinia virus strain WR (VacV-WR, AY243312.1), squirrel poxvirus (SPPV, AY340985.1); genus *Suipox*: Swinepox virus isolate 17077-99 (SwPV, AF410153.1); genus *Capripox*: Sheeppox virus strain A (ShPV, AY077833.1); genus *Molluscipoxvirus*: Molluscum contagiosum virus subtype 1 (MCV, U60315.1); genus *Leporipoxvirus*: Myxoma virus strain Lausanne (MyxV, AF170726.2); genus *Yatapoxvirus*: Yaba monkey tumor virus (Yaba, AY386371.1) and genus *Avipoxvirus*: Fowlpox virus (FwlPV, AF198100.1). The amino acid sequences of the ChPVs were edited to correspond to the amino acid sequences of the PPVs. All phylogenetic analyses were performed with the PHYLIP package version 3.65 (Felsenstein, 2005). Phylogenetic trees were inferred using the maximum-likelihood method. The chosen model of amino acid substitution was the Jones–Taylor–Thornton model with assumed constant rate of change. The maximum-likelihood analyses (with randomized input order and global rearrangements) were performed using the program PROML. The robustness of the phylogenetic analysis and significance of the branch order were determined by 100 data set bootstrap resampling with the programs SEQBOOT and CONSENSE. Additionally, to evaluate the potential role of host range and geographic isolation on the evolutionary divergence of the detected pinniped poxviruses, phylogenetic trees were constructed using only the sequences derived from poxviruses of pinnipeds using the exact methodology outlined above (Figs. 3 and 4).

Nucleotide sequence accession numbers

The nucleotide sequences of SLPV-2, SLPV-3, GSPV-1, HSPV-1 and HSPV-2 generated in this study were deposited in the GenBank data library under the respective accession numbers: DQ273137, DQ273138, DQ273134, DQ273135 and DQ273136.

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