DETECTION AND MOLECULAR CHARACTERIZATION OF CETACEAN AND PINNIPED POXVIRUSES ASSOCIATED WITH CUTANEOUS LESIONS

By

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This document is dedicated to my husband, Ethan Sherman, for his support and patience through these long journeys, and to my loving mother, Camille, who holds my hand along the way.

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DETECTION AND MOLECULAR CHARACTERIZATION OF CETACEAN AND PINNIPED POXVIRUSES ASSOCIATED WITH CUTANEOUS LESIONS

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Poxviruses are widespread and successful pathogens, known to infect a variety of vertebrates including, reptiles, birds, and over 30 species of mammals and several species of insects. Terrestrial poxviruses encompass a variety of well known etiologic agents, that are currently classified into eight genera, within the Chordopoxvirinae subfamily. While significant advances have been made in understanding the genomic sequences of terrestrial poxviruses, little is known about marine poxviruses. DNA extracted from skin lesions of cetaceans in oceanaria and rehabilitation facilities as well as free-ranging cetaceans and pinnipeds was assayed by polymerase chain reaction (PCR). Primers were designed to target gene fragments of three genes: DNA polymerase (DNApol), DNA topoisomerase (DNAtopo) and the major envelope protein (MEP) of poxviruses and parapoxviruses based on numerous DNA sequences available in the National Center for Biotechnology Information (NCBI) database. Targeting of the poxvirus DNApol gene yielded 543-bp fragments when swinepox (SPV) and mule deer poxvirus (MDPV) DNA

were used as templates. Targeting of cetacean poxvirus (CPV) DNA yielded 546-bp amplicons that upon sequencing revealed the existence of two distinct poxvirus sequences that were shown to be approximately 84% and 89% identical in their nucleotides and amino acid sequences, respectively. These findings provide the first evidence of activity of at least two poxviruses in cetaceans, that we provisionally refer to as cetacean poxvirus -1 and 2 (CPV-1 and CPV-2). Amplification of Steller sea lion (Eumetopias jubatus) poxvirus (SSLPV) DNA yielded a 543-bp DNApol gene fragment with nucleotide identity ranges of 76-77% and amino acid identity ranges of 74 -78% when compared to homologous CPV-1 and CPV-2 fragments. Analyses of CPV-1 DNApol fragments showed closest amino acid identity to members of the orthopox genus (~81%), while CPV-2 had identities of ~83%. DNApol fragments amplified from parapox viruses from Steller sea lions, spotted seals (Phoca largha) and a harbor seal (Phoca vitulina) were 536-bp in length and had closest amino acid identities to members of the parapox genus (87-89%). PCR with poxvirus consensus primers that targeted the DNAtopo gene generated 344-bp amplicons using CPV-1, CPV-2, SSLPV, SPV and MDPV DNA as templates. Parapox DNAtopo fragments were amplified from the same set of pinnipeds and were 347, 350 and 252-bp in length. Consensus primers that target the MEP gene of parapoxviruses amplified 594-bp fragments from pinniped para-poxviruses, as well as from pseudocowpox virus DNA. The described molecular assays based on PCR and direct sequencing of amplicons have allowed us to identify several novel poxviruses and investigate the evolutionary relatedness of these viruses when compared to other well known terrestrial poxviruses of vertebrates in the subfamily Chordopoxvirinae.

CHAPTER 1
INTRODUCTION

Introduction to Poxviruses

The family Poxviridae contains the largest known viruses of terrestrial and marine mammals that possess non-infectious, double stranded DNA genomes that range in size from 130-380 kbp and replicate almost exclusively in the cell cytoplasm (Moss, 2001). The basic poxvirus virion incorporates about 100 polypeptides and carries most of the compounds necessary for replication in the host cell. The non-infectious DNA genome (nucleosome) is arranged inside a core membrane that is surrounded by lateral bodies and another outer membrane. Poxviruses encase this outer membrane in yet another lipid bilayer, called the envelope, which functions in host cell attachment (Buller and Palumbo, 1991; Moss, 2001). The Poxviridae is divided into two subfamilies: Entomopoxvirinae, that comprises insect poxviruses, and Chordopoxvirinae, that includes all poxviruses of vertebrates (Moss, 2001). Subfamily Chordopoxvirinae includes eight genera: orthopoxvirus, parapoxvirus, capripoxvirus, suipoxvirus, leporipoxvirus, yatapoxvirus, avipoxvirus and molluscipoxvirus. Poxviruses are highly adapted viruses infecting a large number of hosts, including insects, reptiles, birds, and over 30 mammalian species (Buller and Palumbo, 1991; Moss, 2001; Upton et al., 2003). Some members of the Orthopox genus, such as variola, the causative agent of smallpox virus, are endowed with high virulence and were a scourge to mankind for at least two millennia until its eradication in 1977 (Moss, 2001). Highly invasive and virulent poxviruses of livestock of the Capripoxvirus genus, such as sheep pox, goat pox and

lumpy skin disease viruses, and of the Orthopox genus, such as camel poxvirus, are currently restricted to some parts of the sub-Saharan African continent, the Middle East and the Indian subcontinent (Ireland and Binepal, 1998; Moss, 2001). Poxviruses replicate in the skin and mucosa producing localized or generalized lesions of variable gravity and duration (Buller and Palumbo, 1991). Localized lesions are seen in Molluscum contagiosum, pseudocowpox, and orf virus infections, whereas disseminated lesions are seen in ectromelia, cowpox, monkeypox, and the well known variola virus. (Moss, 2001; Upton et al., 2003). Some parapoxviruses and myxoma virus have the ability to display both disease patterns depending on the animal host (Buller and Palumbo, 1991).

This study, fueled by the generation of genetic data, would not have been possible without the fundamental technique, now implemented in almost every molecular genetics laboratory, the polymerase chain reaction, or PCR. Conceived by Kary Mullis in 1983 (Mullis et al., 1986), the idea revolutionized the fields of biotechnology and molecular biology, paving the way for development of new assays to diagnose medical disorders and a wide range of diseases (Schluger and Rom, 1995). PCR uses simple principles of DNA replication combined with the unique properties of a DNA polymerase from thermophilic bacteria to mimic the DNA replication process in-vitro, exponentially amplifying copies of the targeted DNA (Bej et al., 1991). While the uses of PCR span the range of medical diagnoses, the relevance of this technique to virology is its facilitation of cloning and sequencing of viral genes for the purposes of viral comparison and classification (Bej et al.,, 1991; Ropp et al., 1995; Elnifro et al., 2000). Prior to PCR technology, viruses were isolated in cell culture before any further analyses could occur

(Levine, 2001). Identification and differentiation of viruses before the implementation of PCR relied on less specific serologic assays such as, virus neutralization, hemagglutination and immuno fluorescence assays, later complemented by restriction endonuclease profiles of viral DNA resolved in agarose or polyacrylamide gels (Fleming et al., 1993; Robinson and Mercer, 1995; Ropp et al., 1995; Mangana-Vougiouka et al., 1999; Moss, 2001). These techniques require large amounts of viral DNA typically harvested from virus infected cultures. This approach hindered the study of viruses that do not readily grow in culture, such as papilloma viruses, or for that matter, poxviruses of marine mammals. The advent of PCR allowed for the direct amplification of viral DNA and rapid genome sequencing (Moss, 2001). With the sudden accumulation of genome sequences from numerous viruses representative of most families, detailed examination of genetic relationships escalated coupled with a new understanding of viral taxonomy. For example, upon genetic comparison, it was found that two genetic mutations were responsible for the antigenic difference between feline panleukopenia virus and canine parvovirus (Levine, 2001). Since the late 1980's, PCR protocols have been developed to detect a wide variety of human and animal viruses representing several viral families and genera including: hepatitis, papilloma, influenza, rhabdo-, retro-, herpes-, calici-, adeno- and pox-viruses, among numerous others (De Rossi et al., 1988; Puchhammer-Stockl et al., 1990; Vandenvelde et al., 1990; Sacramento et al., 1991; De Leon et al., 1992; Hondo and Ito, 1992; Morishita et al., 1992; Vesy et al., 1993; Ropp et al., 1995; Heredia et al., 1996; Vantarakis and Papapetropoulou, 1999; Inoshima et al., 2000). Considering poxviruses in particular, PCR and genome sequencing has meant the evolution from sometimes vague histopathologic and electron microscopic (EM) diagnoses to much

more definitive genetic assays for poxvirus infection (Fleming et al., 1993; Ropp et al., 1995; Mangana-Vougiouka et al., 1999; Damaso et al., 2000; Gubser and Smith, 2002; Howsamani et al., 2004) Most poxviruses share a common ovoid or brick-like shape and measure 200 – 400 nm in length with tubules arranged in an irregular pattern on the envelope surface (Buller and Palumbo, 1991; Moss, 2001). The exception to this is the genus parapox, which has a notable criss-cross tubule pattern on the envelope surface (Moss, 2001). Because of this common poxvirus morphology, it is difficult to discern between genera of poxviruses when using techniques like histopathology and EM. However, PCR and sequencing methods reveal not only the genus, but in most cases, species of the virus being examined (Ropp et al., 1995, Damaso et al., 2000; Becher et al., 2002; Howsamani et al., 2004). Beginning in 1990 with the sequence for vaccinia virus- Copenhagen strain (Goebel et al., 1990), complete poxvirus genomes sequences have been generated using techniques derived from PCR, restriction endonuclease digestion, and basic DNA cloning and sequencing. The generation of complete genome sequences in pox virology reached a climax in the years 2000-2002 with the release of 14 complete genome sequences representing six genera within the sub-family Chordopoxvirinae and one in sub-family Entomopoxvirinae (Gubser et al., 2004). Included in these were complete genome sequences of variola, vaccinia, monkeypox, camelpox, fowlpox, lumpy skin disease virus, goatpox, sheeppox, swinepox, and Yaba-like disease virus, as well as, *Amsacta moorei* entomopoxvirus (Goebel et al., 1990; Shchelkunov et al., 1995; Antoine et al., 1998; Shchelkunov et al., 2000; Tulman et al., 2001; Afonso et al., 2002; Gubser and Smith, 2002; Gubser et al., 2004). These sequencing advances have allowed for a better ability to define and understand the

evolutionary relationships between the different poxvirus genera. Comparing genes that have been identified as highly conserved can aid in new virus characterization and comparison. Attempts at obtaining a more global understanding of poxvirus genes and gene families have been made using the Poxvirus Bioinformatic Resouce (PBR; www.poxvirus.org) and have identified 49 conserved gene families in 21 complete poxvirus genomes (Upton et al., 2003). Previous studies have elected phylogenetic analysis to be the best tool available for characterizing poxviruses known to date (Afonso et al., 2002; Becher et al., 2002, Gubser et al., 2004). These studies present an easily comprehendible picture of the ancestral lines of viruses, including possible progenitors for different lineages. The increase in numbers of available sequences permitted attempts to create a more global understanding of poxvirus genetic relationships, mainly through the use of evolutionary analysis. As different software programs emerged and improved over time, numerous phylogenetic analyses have been conducted, initially on single genes or gene fragments (Zanotto et a., 1996; Afonso et al., 2002; McGeoch et al., 2000; Gubser and Smith, 2002; Hosamani et al., 2004; Tryland et al., 2005). Phylogenetic trees constructed using different individual proteins can yield varying topologies, depending on the stringency of conservation of the DNA sequence for the specific gene (Gubser et al., 2004). More recently, phylogenetic studies using large fragments of the central region of the genome or the complete genome sequences have been published, revealing a more complete and accurate picture of poxvirus phylogeny (Fleming et al., 1993; Upton et al., 2003; Delhon et al., 2004; Gubser et al., 2004,). The results of these studies served to verify what had been previously accepted in taxonomic classification of poxviruses by designating the viruses into groups that

corresponded with the accepted poxvirus genera. Gubser et al. (2004) used the sequences of 26 poxviruses representing all poxvirus genera except parapoxvirus, in a phylogenetic study. They found that the general organization of the Chordopoxvirinae genome was conserved, specifically in regard to the central region genes encoding proteins for RNA and DNA replication, virion assembly and structural proteins. This concurs with a previous study by Upton et al. (2003), where authors performed large scale genetic analysis on 21 complete poxvirus genomes. These authors used the Virus Genome Database to identify genes that are most highly conserved among the family Poxviridae, and determined that those genes were involved in DNA replication and transcription. Gubser et al. (2004) used these results to construct a tree incorporating 17 of the 49 proteins that are conserved in all poxviruses, and found that the viruses included in the subfamily Chordopoxvirinae could be divided into four main groups: the Molluscipox genus, the Avipox genus, the Orthopox genus, and a group containing the Yatapox, Capripox, Suipox and Leporipox genera. The tree suggests that the latter group includes viruses evolved more recently, and thus are genetically more related to each other than to the first three groups. It should be noted that the parapoxvirus genus was excluded from this study, and it is speculated that it might clad into its own group, if similar analyses were repeated. Characterization of novel poxviruses such as muledeer poxvirus, spectacled caiman poxvirus, Embu virus and Cantangalo virus that have not yet been assigned to a genus, may be aided by these new techniques. Equally mysterious are the poxviruses that have been known to infect various species of cetacea and pinnipedia. Though often and easily recognized by clinical means, these viruses have not been antigenically or molecularly characterized.

Poxvirus Infections of Cetaceans

Poxviruses have been known (for over 30 years), to affect various cetacean and pinniped species (Wilson et al., 1969; Wilson and Poglayen-Neuwall, 1971; Geraci et al., 1979; Baker, J.R., 1992a,b; Baker and Martin, 1992; Van Bressem et al., 1993). Numerous studies have reported skin lesions associated with poxvirus infections in cetaceans and parapoxvirus infections in pinnipeds (Geraci et al., 1979; Osterhaus et al., 1990; Baker and Martin, 1992; Van Bressem et al., 1993; Nettleton et al., 1995). In cetaceans, poxvirus lesions are described as areas of hyperpigmentation of the skin with pinhole marks, termed "tattoo" or "ring" lesions (Geraci et al., 1979; Van Bressem et al., 1993). These lesions were reported to persist for months to years, and typically regress without treatment (Geraci et al., 1979, Smith, 1983). The appearance of poxviruses lesions in cetaceans seems different from that of poxvirus infections described in terrestrial vertebrates (Geraci et al., 1979). Lesions associated with cetacean poxviruses remain relatively flat, and in some advanced stages, form slightly dark depressions in the center of the lesions (Geraci et al., 1979). Conversely, lesions associated with terrestrial poxviruses may form raised nodules in the skin, and often advance to erupted pustules (Robinson and Mercer, 1995; Damaso et al., 2000; Moss, 2001; Delhon et al., 2004). Geraci etal. (1979), provided an explanation for the difference in clinical appearance and progression of lesions which involves the unique metabolic and mitotic rate of the epidermal cells of the cetacean integument. Further studies on the progression of the disease associated with cetacean poxvirus infection have focused finding histopathological changes with presence of intracytoplasmic inclusions and on morphological characterization of the cetacean poxvirus using electron microscopy (Smith, 1983; Van Bressem, 1993). While the prevalence and conditions that participate

or facilitate the occurrence of poxvirus infection in cetaceans have not been studied, Van Bressem et al., (1993), reported 8.1% and 30% prevalence of tattoo lesions in 74 dusky dolphins (Lagenorhynchus obscurus) and 10 Burmeister's porpoises (Phocoena spinipinnis), respectively, that were examined as fishing by-catch in 1990. The true prevalence of poxvirus infection in wild cetacean populations is unknown; however, Geraci et al., (1979) suggested an association of the occurrence and severity of tattoo lesions, with animals under considerable environmental stress or those exhibiting poor general health. The authors cited specific cases involving captive dolphins afflicted with lesions that improved under less stressful environmental conditions (Geraci et al., 1979).

In contrast to the abundance of sequence data for terrestrial poxviruses, even though the occurrence of poxviruses in marine mammals has been well documented for at least three decades (Wilson et al., 1969; Wilson and Poglayen-Neuwall, 1971; Geraci et al., 1979; Baker, J.R., 1992a,b; Baker and Martin, 1992; Van Bressem et al., 1993), almost no molecular data are available for marine poxviruses. Previous reports have described the pathogenicity and the gross and microscopic lesions after the infection of cetaceans and pinnipeds with marine poxviruses. Most of these infections were diagnosed by demonstrating characteristic poxvirus particles by electron microscopy or the presence of acidophilic intracytoplasmic inclusion bodies in sections of lesions by light microscopy (Flom and Houk, 1979; Geraci et al., 1979; Smith, 1983; Baker, 1992a; Van Bressem et al., 1993). The first published description of poxvirus infection in captive and free-ranging cetaceans discussed ring and tattoo type lesions observed on seven bottlenose (Tursiops truncatus) and one Atlantic white-sided dolphin (Lagenorynchus acutus) (Geraci et al., 1979). Lesions were noted to occur most often on

the dorsal body, dorsal fin and flukes and pectoral flippers (Figure 3-1). Samples of the lesions were examined by light microscopy and electron microscopy, which revealed eosinophilic intracytoplasmic inclusions containing virus particles with typical poxvirus morphology. The condition, termed "dolphin pox", was found to vary in time course, severity and clinical appearance and recurrence. Although the author identifies a poxvirus as the causative agent for the observed lesions, associations between the environmental conditions, general animal health and stress level were also considered as possible reasons for the variations in disease progression. Concurrent studies with similar findings were reported in three more Atlantic bottlenose dolphins (Flom and Houk, 1979). While the pox lesions in both studies are reported to exist without causing any serious harm or consequence to the animals' health, one exceptional case was cited describing a dolphin that died after developing generalized lesions (Flom and Houk, 1979). Smith et al. (1983) reported an observation of two distinct regression patterns of the typical dolphin poxvirus tattoo lesions. The first regression pattern consisted of the lesions becoming raised and edematous that, over time, became depressed and disappeared. The second regression pattern occurred following lesion biopsies where the lesions disappeared in zones surrounding the incision. Samples were taken from both raised and typical flat tattoo lesions, reacted with dolphin sera and evaluated by immunoelectron microscopy. Positive reactivity occurred between the sera and the raised endematous lesions, but not with the flat tattoo lesion. The significance of this study is two fold: it is the first documentation of antibody response to a poxvirus recovered from dolphin lesions and secondly, it suggests the possibility of two antigenically different poxviruses that cause lesions with dissimilar clinical appearance. Over the next ten

years, numerous reports of poxvirus infections in various cetacean species surfaced (Baker, 1992a,b; Baker and Martin, 1992; Van Bressem et al., 1993). Cetacean species in which infections with poxviruses have been previously reported include: Atlantic bottlenose dolphin (Geraci et al., 1979; Flom and Houk, 1979), Atlantic white-sided dolphin (Geraci et al., 1979), common dolphin (Delphinus delphis) (Britt and Howard, 1983), dusky dolphin (Lagenorynchus obscurus) (Van Bressem et al., 1993), striped dolphin (Stenella coeruleoalba) (Baker, 1992a), white beaked dolphin (Lagenorhynchus albirostris) (Baker, 1992a,b) and Hectors dolphin (Cephalorhynchus hectori) (Geraci et al., 1979; Baker, 1992a, b; Van Bressem et al., 1993, 1999). Similarly, poxvirus infections have previously been described in long finned pilot whales (Globocephala melaena) (Baker, 1992a), killer whales (Orcina orca), Burmeister's porpoise (Van Bressem et al., 1993), and harbor porpoises (Phocoena phocoena) (Baker, 1992a,b; Baker and Martin, 1992; Van Bressem et al., 1993, 1999). Routine histological methods continued to provide the best descriptions of microscopic changes in the lesions. Examinations of poxvirus lesions of a Burmeister's porpoise (Phocoena spinipinnis) utilized transmission electron microscopy to highlight an irregular arrangement of tubules on the viral membrane, reminiscent of those seen in orthopoxviruses (Van Bressem et al., 1993). This was the first attempt to characterize the virus in order to assign it to one of the known genera. Little new information has been accumulated in respect to cetacean poxviruses since the early 1990's. The growing availability of DNA sequencing technologies has created opportunities to examine the genome of cetacean poxviruses, and how they situate within the subfamily Chordopoxvirinae.

Poxvirus Infections of Pinnipeds

Poxvirus infections have also been well documented in several pinniped species since 1969. These pinniped poxvirus lesions have a very different appearance from those seen in cetaceans and are typically raised nodules in the skin (Figures 3-2 and 3-3).The first report of seal pox described pox lesions occurring in California sea lions (Zalophus californianus) (Wilson et al., 1969). Closely following that report was one describing an epizootic of a proliferative skin disease among captive California sea lions (Wilson et al., 1972). Histopathology and electron microscopy determined the causative agent to be a poxvirus, and a survey was initiated and sent out to 120 addresses in an attempt to understand more about the scope of this new virus (Wilson et al., 1972). Over the years these methods continued to be employed in identifying sealpox infections of various species including; harbor seals (Phoca vitulina) (Becher et al., 2002, Müller et al., 2003), grey seals (Halichoerus grypus) (Hicks and Worthy, 1987; Osterhaus et al., 1990; Simpson et al., 1994; Nettleton et al., 1995), California sea lions (Wilson et al., 1969), South American sea lions (Otaria byronia) (Wilson and Poglayen-Neuwall, 1971), Weddell seals (Leptonychotes weddellii) (Tryland et al.2005) and northern fur seals (Callorhinus ursinus) (Hadlow et al., 1980).

Because of the similarities to orf and bovine papular stomatitis (BPSV) virion morphology and lesion pathology, the seal poxviruses were designated as probable members of the parapox sub-group (Esposito, 1991). While orf and BPSV were both known to be transmissible to humans (Bowman et al., 1981; Meechan and Macleod, 1992; Delhon et al., 2004), the zoonotic potential of any marine mammal poxvirus was unknown. In 1987, a case report described two seal handlers that developed lesions on their hands similar to milker's nodules that occurred while working with grey seals with

typical seal pox lesions (Hicks and Worthy, 1987). Healing times varied and one handler experienced relapses over the next several months. Negative staining of the virions from both the seals and the handlers suggest that the handlers' nodular lesions were caused by the seal pox virus.

The lesions described in South American sea lions were distinct from those previously described in California sea lions and harbor seals (Wilson and Poglayen-Neuwall, 1971). The described lesions in California sea lions and harbor seals proliferate outward, but the lesions of the South American sea lions proliferate downward into the dermal layer. The intracytoplasmic inclusion bodies differed in morphology being large and oval shaped versus small and irregular. In addition, the virion of the South American sea lion pox virus appeared rectangular or brick shaped, versus the elongated or cylindrical shape normally associated with previous reports of seal pox virus shape. The results of this report suggested the existence of two poxviruses with the ability to infect pinnipeds. Similar observations were made in the reexamination of old formalinized samples from a stranded northern fur seal pup (Hadlow et al., 1980). Tissues preserved from an animal that was necropsied in 1951 were examined for poxvirus and found to resemble those reported in South American sea lions more than in California sea lions or Harbor seals. The suggestion of the existence of two pinniped poxviruses resurfaced in a report that outlined the isolation of both parapox and orthopox-like viral particles from lesions of a grey seal (Osterhaus et al., 1990). The in vitro culture of the orthopox-like virus was apparently, only possible in primary grey seal skin cells. However. no reports on the characterization of this poxvirus has appeared since. A parapoxvirus was later

isolated from grey seal pox lesions using primary grey and harbor seal kidney cells (Osterhaus et al., 1994, Nettleton et al.,1995).

The first mention of using the polymerase chain reaction (PCR) to test for pinniped poxvirus infection surfaced in 2002 (Becher et al., 2002). The PCR primers used were known to direct the amplification of a segment of the major envelope protein gene and had been reported as a diagnostic tool for parapox infections of cattle, sheep and Japanese serows (Inoshima et al., 2000). Skin lesions from harbor seals were analyzed for parapoxvirus infection. Nucleotide and amino acid sequences obtained from the DNA sequence of the amplified PCR fragments were compared against those of BPSV, pseudocowpox virus (PCPV), parapoxvirus of red deer in New Zealand (PVNZ) and orf virus (OV) and found to be significantly different in both cases: <79% nucleotide identity and <77% amino acid identity. The authors suggested that the seal parapoxviruses constituted a separate species within the genus Parapoxvirus (Becher et al., 2002). Presently, "sealpox" is classified as a tentative member of the parapox genus. It is evident that, while many advances have been made since the early days of poxvirus detection in marine mammals, much is still unknown about the genomic organization and evolutionary relationships of these viruses.

CHAPTER 2
MATERIALS AND METHODS

Sample Acquisition

Fresh and frozen skin lesions from 109 stranded, free-ranging and captive marine mammals were harvested and shipped to our laboratories between January, 2001 and March, 2005 for analyses of poxvirus infection. Lesion scrapings and biopsies from captive marine animals were provided by several amusement parks and aquariums from Florida, Texas, Portugal, and Hong Kong. Tissues from stranded and free-ranging animals were obtained from numerous participants of the Southeast, Northeast and Alaska Stranding Networks, as well as the Alaska Department of Fish and Game. All samples collected from stranded marine mammals were obtained by licensed personnel from the Networks. These lesions were obtained from 92 cetaceans and 17 pinnipeds. Donor species were: Forty-two Atlantic bottlenose dolphin (Tursiops truncatus), twenty-two bowhead whales (Balaena mysticetus), seven Indopacific bottlenose dolphin (Tursiops aduncus), four rough-toothed dolphin (Steno bredanensis), four pygmy sperm whales (Kogia breviceps), two killer whales (Orcina orca) (Dover, 1992), two short-finned pilot whales (Globicephala macrorhynchus), three Risso's dolphin (Grampus griseus), one striped dolphin (Stenella coeruleoalba), one Pacific white-sided dolphin (Lagenorhynchus obliquidens*)*, one dwarf sperm whale (Kogia sima), one spinner dolphin (Stenella longirostris), one Pantropical spotted dolphin (Stenella attenuata), one Harbor porpoise (Phocoena phocoena), fourteen Steller sea lions (Eumetopias jubatus), two spotted seals (Phoca largha), and one harbor seal (Phoca vitulina).

Histopathology and Electron Microscopy

A 6-mm punch biopsy was taken of two Steller sea lion skin lesions. One half of the biopsy was placed in 10% neutral buffered formalin and the other half frozen in dry ice and stored at –70º C for DNA extraction and PCR analysis. Formalin fixed samples were embedded in paraffin, sectioned at 5 µm, and stained with hematolyxlin and eosinophilic for evaluation by light microscopy. Negative staining electron microscopy was also performed on formalin-fixed specimens. The samples were homogenized in distilled water in a Ten-Broeck grinder, clarified by centrifugation at 4,000xg for 5 min, the supernatant removed to a clean tube and centrifuged at 12,000xg for 1 hr. The pellet was resuspended in 2% phosphotungstic acid solution at pH 6.8 containing 0.01% bovine serum albumin and a drop of this suspension was applied to a carbon coated formvar film on a 400 mesh copper grid and the excess wicked away. The grid was examined with a Zeiss EM 109 microscope (Carl Zeiss, Inc., Thornwood, New York, USA).

Extraction of Total DNA

All samples were processed to obtain total DNA using the DNeasy kit (Qiagen, Valencia, California, USA) according to the protocol indicated by the manufacturer. Briefly, 25 mg of tissue was cut into small pieces and combined with 180 µl of lysis buffer ATL and 20 µl of proteinase K. The tissues were incubated at 55°C until lysis was complete. DNA was precipitated by the addition of 200 µl absolute ethanol and spun through the DNeasy Spin Column. After two washes with buffers AW1 and AW2, the DNA was eluted in 200 µl buffer AE. The quality and content was evaluated by spectrophotometry using the Ultrospec 3000 (Amersham Biosciences Corp., Piscataway, New Jersey, USA). Each group of tissue samples was extracted along with a known negative sample to be used as a negative control for analysis.

General Conditions for PCR

Reaction tubes for PCR contained 200 nM of each primer, 100 μM of each deoxynucleoside triphosphate (dNTP), 10 mM KCl, 10 mM $(NH_4)_2 SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1% Triton X-100 at pH 8.8, 1 unit of Taq DNA polymerase (New England BioLabs, Beverly, Massachusetts, USA), and 500 ng of DNA template, in a final volume of 50 μl. All PCR cyclings were performed in a PTC-100 thermal cycler (MJ Research, Inc., Waltham, Massachusetts, USA). Cycling conditions for the amplification of the DNA polymerase and DNA topoisomerase gene fragments of poxviruses were: Initial denaturation at 94°C for 1 min, followed by 39 cycles, each comprising of a denaturation step at 94°C for 30 sec, an annealing step at 45°C for 30 sec, and an elongation step at 72°C for 30 sec. The last cycle included an extended elongation step at 72°C for 10 min. Cycling conditions for the amplification of the DNA polymerase gene of parapoxviruses were similar, except that the annealing temperature for the parapoxviruses was 61^{o}C. The cycling conditions for the amplification of the DNA topoisomerase gene fragments of parapoxviruses from Steller sea lions, harbor seals and spotted seals were also similar; however, the annealing temperatures were 53^{o}C, 51^{o}C and 58^{o}C, respectively.

Poxvirus PCR Targeting the DNA Polymerase Gene

Oligonucleotide primers that target sequences within the DNA polymerase gene were designed based on sequences of lumpy skin disease virus (LSDV) and swinepox virus (SPV) deposited in the GenBank database and from mule deer poxvirus (MDPV) sequenced in our laboratories (Accession number AY841895). These sequences were: Forward primer CR 422: 5'– ATA CAG AGC TAG TAC ITT AAT AAA AG - 3'and

reverse primer CR 421: 5'- CTA TTT TTA AAT CCC ATT AAA CC - 3'. MDPV or SPV DNA was used as a positive control, yielding DNA fragments of 543 base pairs (543-bp) in size. Negative tissues were used as negative controls.

Poxvirus PCR Targeting the DNA Topoisomerase I Gene

Oligonucleotide primers were designed based on the sequences of homologous genes of LSDV, SPV and MDPV (AY841896). The primer sequences were: CR 432: 5'– TAA TGG AAA CAA GTT TTT TTA T – 3' and CR 433: 5'– CCA AAA ATT ATA TAA AAA CG – 3'. These primers directed the amplification of a 344-bp DNA fragment when SPV and MDPV genomic DNA was used as a positive control. Negative tissues were used as negative controls.

Poxvirus PCR Targeting the Major Envelope Gene

Oligonucleotide primers were designed based on the sequences of vaccinia, camelpox, monkeypox, variola, ectromelia and cowpox. Two forward primers were designed, the first one included the gene start codon, and the second was 42-bp internal to the start codon. The two forward primer sequences were: CR 597: 5' – ATG TGG CCA TTT RYA TCR GY -3' and CR 598: 5' – CTG GTA GAA ACA CTA CCA GAA AAT - 3'. The reverse primer sequence includ the stop codon and was designed as follows: CR596: 5'- TTA AAT TTT YAA CGA TTT ACT GTG GC -3'. The expected sizes of the fragments generated by these primers were 1118-bp and 1076-bp respectively Vaccinia virus DNA was used as a positive control. Negative tissues were used as negative controls..

Poxvirus PCR Targeting the Hemagglutinin Gene of Orthopoxviuses

PCR primers were designed to target the Hemagglutinin (HA) gene of orthopoxviruses based on the sequences of camelpox, vaccinia, monkeypox, cowpox,

variola, and ectromelia. The primers target the full HA gene and predict the amplification of a 1138-bp fragment from orthopox viruses. The primers were forward primer CR 619: 5'- GAT TTT CTA AAG TRY TTG GAR AGT TTT AT- 3' and reverse primer CR620: 5'-GCT GTC TTT CCT IAA CCA GAT G -3'. Vaccinia virus DNA was used as a positive control. DNA extracted from a negative tissues and a negative tube containing no DNA were used as negative controls.

A previously described set of primers was also used to amplify the HA gene sequence of orthopoxviruses (Damaso et al., 2000).

Parapoxvirus PCR Targeting the DNA Polymerase Gene

Oligonucleotide primers that target genomic sequences within the DNA polymerase gene of parapoxviruses were designed based on genomic sequences of orf (NC_005336) and bovine papular stomatitis (NC_005337) viruses that exist in the GenBank database. These primer sequences were: CR 54l: 5'- GCG AGC ACC TGC ATC AAG - 3'; CR 540: 5'- CTG TTI CGG AAG CCC ATG AG - 3'. Pseudocowpox virus DNA was used as a positive control. Negative tissues were used as negative controls..

Parapoxvirus PCR Targeting the DNA Topoisomerase I Gene

Oligonucleotide primers were first designed based on the nucleotide sequences of the orf (NC_005336) and bovine papular stomatitis (NC_005337) virus DNA topoisomerase gene sequences from the GenBank database. These primers were: CR 550: 5' - TCA TGG AGA CSA GCT TCT TCA TC - 3'(forward); CR 551: 5'- CCA GAA GTT GTA CAR RAA SGT GTA G - 3'(reverse). This primer set, however, did not amplify parapoxvirus sequences from DNA extracted from lesions of all species of marine mammals tested. Thus, a second primer set was designed based on sequences obtained from the Steller sea lion parapoxvirus DNA topoisomerase gene fragment. The

primer sequences were: CR 557: 5’ – TCA TGG AGA CGA GCT TCT TCA TC – 3’(forward); CR 558: 5’ – CCA GAA GTT GTA CAA GAA GGT GTA G – 3’(reverse). As these two sets of primers still did not amplify parapoxvirus DNA from spotted seals, a third set of primers had to be designed after performing a line up between the Steller sea lion and harbor seal parapoxviruses DNA topoisomerase gene fragments. These primer sequences were: CR 570: 5’ - GTC YTT AAC GCG AAT RCC AAA GC - 3’(forward); CR 571: 5’- AGC GGM ACW GTK GGY TTG CTC AC - 3’(reverse). Pseudocowpox virus DNA was used as a positive control. Negative tissues were used as negative controls..

Parapoxvirus PCR Targeting the Major Envelope Protein Gene

PCR was performed using previously published consensus primers known to target the major envelope protein gene of parapoxviruses (Inoshima et al., 2000). These primers were: FP-PPP-4: 5’- TAC GTG GGA AGC GCC TCG CT-3’(forward); RP-PPP-1: 5’-GTC GTC CAC GAT GAG CAG CT-3’(reverse). This primer set directs the amplification of a 594-bp DNA fragment. Pseudocowpox virus DNA was used as a positive control. Negative tissues were used as negative controls..

Gel Electrophoresis

Amplified DNA fragments were resolved by horizontal electrophoresis of 20-30 µl of the PCR product in 1.0% agarose containing ethidium bromide (0.5 µg/ml), visualized under ultraviolet light and photographed using a gel documentation system (Bio-Rad Laboratories, Inc., Hercules, California, USA).

Cloning of amplified DNA fragments

To obtain the complete nucleotide sequence of all amplified DNA fragments, these were cloned into the bacterial plasmid vector pCR 2.1 TOPO TA (Invitrogen, Carlsbad,

California, USA) following the manufacturer's protocol. Competent E. coli DH5 alpha cells were transformed with vector-insert reactions and streaked on 2XYT agar medium containing ampicillin (100 μg/ml). Colonies were grown overnight as minicultures, in 3 ml of 2XYT medium containing ampicillin (100 μg/ml), while shaken at 270 rpm at 37°C. Plasmid DNA was extracted from 1.5 ml of the minicultures using a phenol-free method (Zhou et al., 1990). To screen for recombinant plasmids, plasmid DNAs were digested with restriction enzymes HindIII, EcoRI, ApaI, BamHI, and a combination of enzymes ApaI and BamHI. Recombinant plasmids containing the correct insert were purified for sequencing using the Aurum™ Plasmid Mini Kit or the Plasmid Midi-Prep Kit (Bio-Rad Laboratories Inc., Hercules, California, USA) according to the manufacturer's protocol. In brief, this involved first pelletting bacteria from 1.5 ml of bacterial culture by centrifugation at 13,000 rpm for 30 seconds and then resuspending and lysing the pellet in the supplied buffer. A neutralization buffer was then added, and the cell debris was pelleted via centrifugation at 13,000 rpm for 10 minutes. The cleared supernatant was harvested and spun through the supplied column and washed with the wash solution provided. Purified plasmid DNA was eluted in 50 μl of elution solution, also provided in the kit.

DNA Sequencing and Sequence Analysis

Amplified DNA fragments that were strong and uncontaminated with other fragments as observed after gel electrophoresis were purified using the Wizard SV Gel and PCR Clean-up System (Promega Corporation, Madison, Wisconsin, USA). This protocol involved adding an equal volume of membrane binding solution to the PCR product and purifying the DNA by centrifugation through the supplied column. The

column was washed with wash solution twice, and the DNA was eluted in 50 µl of nuclease free water, quantified by spectrophotometry, and sequenced directly. Between 50–100 fmol of purified PCR products were sequenced in duplicate in both directions using specific forward and reverse primers and the proprietary chemistry for the CEQ 2000XL sequencing instrument (Beckman-Coulter Inc., Fullerton, California, USA). Chromatograms were manually reviewed for potential misreadings using the Chromas 2.3 software (Technelysium Pty Ltd., Tewantin, Queensland, Australia) and exported into the Seqed function of the University of Wisconsin Package Version 10.2 (Genetics Computer Group - GCG, University of Wisconsin, Madison, Wisconsin, USA).

Sequences were analyzed using the Gap, Translate and Lineup functions of this software and assembled using SeqMan, SeqEd and MegAlign (DNAStar, Lasergene software, Madison, Wisconsin, USA). The BLAST function of the National Center for Biotechnology Information (NCBI) was used to identify poxvirus sequences most closely related to those of marine mammal poxviruses. Neighbor-joining phylogenetic trees were generated by PAUP 4.0 (Sinauer Associates, Sunderland Massachusette, USA) software, using ClustalW slow and accurate function using Gonnet residue weight table, gap penalty of 11 and gap extension penalty of 0.2. The trees were based on the amino acid sequences deduced from the homologous DNA fragments of the DNA polymerase and DNA topoisomerase genes from members of the Chordopoxvirinae subfamily of poxviruses obtained from the GenBank repository through the NCBI website. The GenBank accession numbers (in parentheses) for the viral sequences used in the genetic analysis were: Lumpy skin disease (AF409137), sheeppox (NC_004002), goatpox (AY077835), swinepox (NC_003389), canarypox (AY318871), cetacean poxvirus-1

(AY463004-AY463007), cetacean poxvirus-2 (AY846759, AY846760), fowlpox (NC_002188), Steller sea lion pox (AY424954, AY424955), harbor seal parapox (AY952937-AY952939, AF414182), spotted seal parapox (AY780676, AY780677, AY780678), Steller sea lion parapox (AY952940-AY952984), Weddel sealpox (AJ622900), camelpox (AF438165), variola (NC_001611), rabbitpox (AY484669), monkeypox (NC_003310), mule deer pox (AY841895, AY841896), vaccinia (AY243312), ectromelia (NC_004105), pigeonpox (M88588), red deer parapox (AB044794), cowpox (AF482758), Yaba monkey tumor (AY386371), rabbit myxoma (NC_001132, AAF14910), rabbit fibroma (NC_001266), orf (NC_005336), bovine papular stomatitis (NC_005337) and molluscum contagiosum (NC_001731).

Primer Specificity and Sensitivity Assays

The poxvirus DNA polymerase and DNA topoisomerase PCR assays were applied to swinepox, pseudocowpox, muledeerpox, CPV-1, CPV-2, SSLPV, HSPPV, SSPPV and SSLPPV DNA to determine primer specificity. Ten-fold serial dilutions ranging from 100 ng to 0.001 fg of pCRII-Topo 2.1 plasmid that contained the amplified 546-bp CPV-1 DNA polymerase fragment or 344-bp DNA topoisomerase fragment were PCR amplified using primer set CR421/CR 422 and primer set CR432/CR433, respectively, to define the general sensitivity of these assays.

The parapoxvirus DNA polymerase, DNA topoisomerase, and major envelope protein gene PCR assays were applied to pseudocowpox, CPV-1, CPV-2, SSLPPV, HSPPV, and SSPPV DNA to determine the primer specificity. Ten-fold serial dilutions ranging from 100 ng to 0.001fg of pCRII-Topo 2.1 plasmid containing the parapox DNA polymerase, DNA topoisomerase, or major envelope protein gene fragments, were PCR

amplified using the respective primer set to determine the general sensitivity of the assays.

Virus Isolation

Numerous attempts to isolate pox and parapox viruses from marine mammal skin lesions were made. Fresh or frozen tissue sample were homogenized in a 2 ml glass Tenbroeck tissue grinder. One, five and ten percent dilutions were made using Dulbecco's modified medium (DMEM) containing antibiotic/antimycotic drugs. The dilutions were clarified via centrifugation at high speed (13,000 rpm for 1 minute) to reduce bacterial contamination. Tissue culture lines that were utilized in virus isolation attempts included: African green monkey kidney (Vero), Madin- Darby canine kidney (MDCK), Tursiops trucatus lung (TurtruLu), Tursips trucatus kidney (TurtruK), Phoca vitulina ovary (PhovituOv), and Phoca vitulina lung (PhoVitLu). Dilutions made from PCR positive cetacean and SSL pox skin lesions were innoculated onto Vero, MDCK, TurtruLu and TurtruK cell cultures. Dilutions made from pinniped pox and parapox lesions were innoculated on Vero, MDCK, PhovitO ad PhovitLu cell cultures. The inoculum was allowed to adsorb onto the cell monolayers for 2-3 hours, after which the monolayers were carefully rinsed with DMEM, fed with DMEM supplemented with 1.0 – 5.0% fetal bovine serum and then incubated 37°C in an atmosphere of 5% CO_2. Innoculated and non-innoculated cultures were checked daily for cytopathic effects (CPE), the maintenance medium was changed as needed, and discarded after 14 days, or two passages if no CPE was observed.

CHAPTER 3
RESULTS

Summary of Positive Samples

Out of 109 fresh and frozen skin lesion samples tested, poxvirus positive results were determined for 10 cetacean lesions including; four Indo-Pacific bottlenose dolphins, two rough-toothed dolphins, one striped dolphin, two Atlantic bottlenose dolphin and one bowhead whale (Figure 3-1). Three Steller sea lion skin samples also tested positive for poxvirus (Figure 3-2). Assays for parapoxvirus yielded six positive results including lesions from three Steller sea lions, two spotted seals, and one harbor seal (Figure 3-3).

Histopathology and Electron Microscopy

Skin lesions of two Steller sea lion pups were analyzed using histopathology and electron microscopy. Histology revealed a mass lesion within the dermis composed of large, polygonal epithelial cells. The mass was composed of broad cords of polygonal to round epithelial cells with sharply delineated cytoplasmic borders. The nuclei were consistent in size, round to oval with 1- 2 prominent nucleoli / nucleus, fine granular chromatin and 0-4 mitotic figures/high power field. Some nuclei contained 1-2 clear vacuoles. Many of these epithelial cells contained a single large brightly eosinophilic inclusion body (Figure 3-4). Scattered lymphocytes, plasma cells and neutrophils were present in the dermis surrounding the mass.

On electron microscopy, virus particles were smooth, rounded rectangles approximately 350 x 270 nm consistent with published reports of orthopox viruses (Moss, 2001) (Figure 3-5).

Detection of Poxviruses Targeting the DNA Polymerase Gene

Total DNA extracted from 10 cutaneous lesions from cetaceans and two lesions from Steller sea lions contained poxvirus genomic DNA as evidenced by the amplification of DNA polymerase gene fragments of the expected size. Positive donor cetacean species were: Four Indo-Pacific bottlenose dolphins, two rough-toothed dolphins, one striped dolphin, two Atlantic bottlenose dolphin and one bowhead whale. Similarly, lesions harvested from three Steller sea lion pups also contained amplifiable poxvirus DNA polymerase gene sequences (Figure 3-6). This PCR assay detected the DNA polymerase gene fragments of muledeer poxvirus, swinepox virus, cetacean poxvirus-1 (CPV-1), cetacean poxvirus-2 (CPV-2) and Steller sea lion poxvirus (SSLPV), but did not amplify the DNA polymerase gene fragments of pseudocowpox, Steller sea lion parapoxvirus (SSLPPV), harbor seal parapoxvirus (HSPPV), or spotted seal parapoxvirus (SSPPV). Serial ten-fold dilutions from 100 ng to 0.001fg of Topo 2.1 plasmid containing the CPV-1 DNA polymerase gene fragment were PCR amplified with primers CR 421 and CR422. The minimal amount of CPV-1 DNA detected was 1.0 fg.

Detection of Poxviruses Targeting the DNA Topoisomerase I Gene

A total of seven lesions from cetaceans yielded positive PCR results when the poxvirus DNA topoisomerase gene was targeted. Positive cetacean species were: Two rough-toothed dolphins, two striped dolphins, one Indo-Pacific bottlenose dolphin, one Atlantic bottlenose dolphin and one bowhead whale. DNA fragments corresponding in size to the DNA topoisomerase gene fragments were also amplified from total DNA extracted from lesions of three Steller sea lion pups (Figure 3-7). This PCR assay detected the DNA topoisomerase gene fragments of muledeer poxvirus, Swinepox virus, CPV-1, CPV-2 and SSLPV, but did not amplify the DNA topoisomerase gene fragments

of pseudocowpox, SSLPPV, HSPPV, or SSPPV. Serial ten-fold dilutions from 100 ng to 0.001fg of Topo 2.1 plasmid containing the CPV-1 DNA toposiomerase fragment were PCR amplified with primers CR 421 and CR422. The minimal amount of CPV-1 DNA detected was 1.0 fg.

PCR Targeting the Major Envelope Protein Gene of Orthopoxviruses

PCR was used to target the major envelope protein gene of cetacean and pinniped poxviruses. While the primers amplified bands of the expected size, of approximately 1118-bp using cetacean poxvirus DNA, sequencing of the DNA fragments yielded non-poxvirus DNA sequence. When vaccinia virus DNA was used as template, the same primers drove the amplification of a fragment of the expected size (Figure 3-8).

PCR Targeting the Orthopoxvirus Hemagglutinin Gene

Cetacean and Steller sea lion poxvirus DNA templates were tested using primers designed to amplify the HA gene of orthopox viruses. Although the primers did not detect the presence of the HA gene in either cetacean, or Steller sea lion DNAs, the vaccinia virus DNA positive control validated the PCR protocol amplifying a band at the expected size of 1138-bp (Figure 3-8).

Detection of Parapoxviruses Targeting the DNA Polymerase Gene

Parapoxvirus DNA polymerase gene fragments of the approximate expected size were amplified from total DNA extracted from biopsied or scraped skin lesions of pinnipeds. Donor species that yielded positive results were: Three Steller sea lions, two spotted seals and one harbor seal (Figure 3-9). This PCR assay detected the DNA polymerase gene fragments of pseudocowpox, SSLPPV, HSPPV, or SSPPV, but did not amplify the DNA polymerase gene fragments of muledeer poxvirus, swinepox virus, CPV-1, CPV-2 and SSLPV. Serial ten-fold dilutions from 100ng to .001fg of Topo 2.1

plasmid containing the SSPPV DNA polymerase gene fragment were PCR amplified with primers CR 540 and CR541. The minimal amount of target DNA detected was 0.1 fg (Figure 3-10).

Detection of Parapoxviruses Targeting the DNA Topoisomerase I Gene

PCR targeting the DNA topoisomerase gene of parapoxviruses using the first set of primers (CR550 and CR551) amplified DNA fragments approximately 350-bp in length when total DNA extracted from lesions of Steller sea lions was used as template (Figure 3-11). However, these primers did not amplify DNA topoisomerase gene fragments from lesions of harbor or spotted seals. Serial ten-fold dilutions from 100 ng to 0.001fg of Topo 2.1 plasmid containing the SSLPPV DNA topoisomerase gene fragment were PCR amplified with primers CR 550 and CR551. The minimal amount of target DNA detected was 1.0 fg (Figure 3-12). A second set of primers (CR557 and CR558) was designed based on the Steller sea lion parapoxvirus DNA topoisomerase I sequence that successfully directed the amplification of a fragment of the expected size from the harbor seal parapoxvirus lesion, but not from the spotted seal lesion (Figure 3-11). Serial ten-fold dilutions from 100 ng to 0.001fg of Topo 2.1 plasmid containing the HSPPV DNA topoisomerase fragment were PCR amplified with primers CR557 and CR558. The minimal amount of target DNA detected was 0.1 fg (Figure 3-13). A third set of internal consensus primers (CR570 and CR571) based on the Steller sea lion and harbor seal parapoxvirus DNA topoisomerase I fragment sequences directed the amplification of a fragment of approximately 250-bp from the spotted seal lesions (Figure 3-14). Serial ten-fold dilutions from 100 ng to 0.001fg of Topo 2.1 plasmid containing the SSPPV DNA topoisomerase fragment were PCR amplified with primers CR 570 and CR571. The minimal amount of target DNA detected was 1.0 fg (Figure 3-15).

Detection of Parapoxviruses Targeting the Major Envelope Protein Gene

Oligonucleotide primers PPP-1 and PPP-4 (Inoshima et al.2000) known to amplify a 594-bp fragment within the major envelope gene of parapoxviruses of herbivores and harbor seals, directed the amplification of DNA fragments of similar size using total DNA extracted from skin lesions harvested from three Steller sea lions, two spotted seals and one harbor seal (Figure 3-16). Serial ten-fold dilutions from 100 ng to 0.001fg of Topo 2.1 plasmid containing the HSPPV DNA topoisomerase fragment were PCR amplified with primers CR339 and CR340. The minimal amount of target DNA detected was 0.1 fg (Figure 3-17).

Sequencing and Genetic Analysis

DNA Polymerase

Sequencing of amplified poxvirus DNA polymerase gene fragments from lesions of 12 marine mammals revealed that the fragments were 546-bp in length from 10 cetacean samples representing five species, while those amplified from two Steller sea lion lesions were 543-bp. Sequencing of DNA fragments corresponding to the DNA topoisomerase I gene of poxviruses contained in lesions of cetaceans and Steller sea lions were 344-bp in length. Primers CR541 and CR540 directed the amplification of DNA fragments 536-bp in length, from the DNA polymerase gene of parapoxviruses contained in lesions of three Steller sea lions, two spotted seals and one harbor seal. Targeting of the DNA topoisomerase I gene of parapoxviruses with primers, CR550 and CR551, amplified DNA fragments of 347 or 350-bp when total DNA from lesions from three Steller sea lions was used as template. The second set of primers, CR557 and CR558, also directed the amplification of 350-bp DNA fragments when total DNA from the lesions of a third Steller sea lion and a harbor seal was used as a template. The third set of primers, CR570

and CR571, directed the amplification of DNA topoisomerase I gene fragments 252-bp in length from parapoxvirus lesions from two spotted seals. Targeting of the major envelope protein gene of parapoxviruses with primers validated with ruminant parapoxviruses (Inoshima et al., 2000) and harbor seal parapoxviruses (Becher et al., 2002; Müller et al., 2003), confirmed the universality of these primers for the amplification of parapoxvirus DNA contained in skin lesions of pinnipeds; in this case, Steller sea lions, spotted and harbor seals.

Genetic analysis of the nucleotide sequences obtained from the DNA polymerase gene fragments of poxviruses of cetaceans (546-bp) demonstrated that nine of the 10 nucleotide sequences derived from cetacean poxviruses, shared identities greater than 93.0 and 97.2% at the nucleotide and amino acid level, respectively. We have tentatively grouped these nine poxviruses within a single group that we, herein refer toas cetacean poxvirus-1. The remaining cetacean poxvirus sample derived from a bowhead whale lesion was shown to be at least 84 and 89% identical at the nucleotide and amino acid level, respectively, when compared to homologous sequences from the other nine cetacean poxvirus-1 sequences (Tables 3-1 – 3-3). This virus was being provisionally named as cetacean poxvirus-2. The DNA polymerase gene fragments (543-bp) amplified from cutaneous lesions of two Steller sea lion pups were 100% identical to each other, and at least 76 and 81% identical at the nucleotide and amino acid level, respectively, when compared to homologous sequences of cetacean poxvirus-1. Similar comparisons to the homologous fragments from the bowhead whale (cetacean poxvirus-2) showed identities of 77 and 83% (Table 3-1 – 3-3). Genetic analysis of DNA polymerase gene fragments amplified from skin lesions of pinnipeds associated with parapoxviruses

showed that the viruses contained in these lesions were members of the parapoxvirus genus and showed nucleotide and amino acid identities greater than 98% when compared among themselves. Nucleotide and amino acid sequence comparisons of the DNA polymerase gene fragments of the Steller sea lion poxvirus and the Steller sea lion parapoxviruses showed, respectively, identities of 55 and 61 %. The DNA polymerase fragments obtained from the cetacean and pinniped poxvirus DNA templates were compared with homologous fragments from other terrestrial poxviruses. These numerous pairwise comparisons were made to represent the nucleotide identity, amino acid identity and amino acid similarity (Tables 3-1 – 3-3) of all these viruses. Multiple alignments were performed using the DNA polymerase fragments of CPV-1, CPV-2, SSLPV, SSLPPV, SSPPV, HSPPV (Figures 3-18 and 3-19). The multiple alignment comparing the CPV-1 and CPV-2 DNA polymerase fragments showed a clear difference between the CPV-1 and CPV-2 amino acid sequences (Figure 3-18). The multiple alignment comparing the SSLPV, SSLPPV, HSPPV, and SSPPV DNA fragments showed a clear difference between the pox and parapoxvirus amino acid sequences (Figure 3-19).

DNA Topoisomerase I

Genetic analysis of the DNA topoisomerase gene fragments (344-bp) of cetacean poxvirus demonstrated that six of the seven positive samples had nucleotide and amino acid identities of at least 92 and 95%, respectively (Tables 3-4, 3-5). These six poxviruses had all been included in the cetacean poxvirus-1 type based on sequences of the DNA polymerase gene fragment. The seventh poxvirus corresponding to the bowhead whale poxvirus sample, had identities of 84 and 85% at the nucleotide and amino acid levels when compared to homologous sequences of cetacean poxvirus-1 (Tables 3-4 – 3-6). Based on sequences of the DNA polymerase gene fragment, the

bowhead whale virus has been provisionally named cetacean poxvirus-2. Poxvirus DNA topoisomerase fragments amplified from lesions of the three Steller sea lion pups were identical to each other with about 71 and 75% identity at the nucleotide and amino acid level, respectively, to homologous sequences of cetacean poxviruses-1. Similar comparison to homologous sequences of cetacean poxvirus-2 revealed identities of 72 and 77%. Genetic analysis of the DNA topoisomerase fragments of the Steller sea lion, spotted seal and harbor seal parapoxviruses demonstrated that they belong to the parapoxvirus genus.

Pair-wise comparisons between the DNA topoisomerase gene fragment sequences of the poxvirus from the two Steller sea lion pups and the homologous fragments from the Steller sea lion parapoxviruses showed identities of 52-54 and 57% at the nucleotide and amino acid levels, respectively, clearly demonstrating that these viruses are distinct members of separate genera within the Chordopoxvirinae subfamily (Tables 3-4 - 3-6). The genetic diversity of parapoxviruses of pinnipeds is reflected in the findings that the DNA polymerase gene fragment sequence the Steller sea lion and its homologue in the harbor seal parapoxviruses share 80-98 and 87-98% identity at the nucleotide and amino acid levels, respectively. This identity shows a similar pattern of about 80-99 and 88-99% in the case of the spotted seal parapoxvirus. The DNA topoisomerase gene fragment of the harbor seal parapoxviruses share 96 and 95% identities when compared to the homologous sequence from the spotted seal parapoxvirus. The DNA topoisomerase fragment sequences obtained from the cetacean and pinniped poxvirus DNA templates were compared with homologous fragments from other terrestrial poxviruses deposited in the GenBank database (Tables 3-4- 3-6). These numerous

pairwise comparisons established the nucleotide identity, amino acid identity and amino acid similarity among the most relevant poxviruses. Multiple alignments were generated using the DNA topoisomerase gene fragment sequences of CPV-1, CPV-2, SSLPV, SSLPPV, SSPPV, HSPPV (Figures 3-15 and 3-18). The multiple aligment of the CPV-1 and CPV-2 DNA topoisomerase fragments demonstrates a clear difference between the respective CPV-1 and CPV-2 amino acid sequences (Figure 3-20). The multiple alignment of the SSLPV, SSLPPV, HSPPV and SSPPV DNA topoisomerase gene fragments demonstrates a clear difference between the pox- and parapox- virus amino acid sequences (Figure 3-21).

Major Envelope Protein Gene

Sequence comparisons were performed with the nucleotide and deduced amino acid sequences of the major envelope gene fragments of the various pinnipeds parapoxviruses. Nucleotide and amino acid sequences from the Steller sea lion major envelope fragment were, respectively, 93 and 98% identical to the homologous sequences from the harbor seal parapoxvirus and 93 and 96% identical to the homologous sequences of the spotted seal parapoxviruses (Tables 3-6 – 3-9). Sequences of the major envelope protein gene fragments obtained from the pinniped parapoxviruses were entered into a multiple alignment for simplified comparison (Figure 3-22).

Phylogenetic Analysis

Phylogenetic trees created using the amino acid sequences of various species of marine mammal pox and parapox virus sequences plus numerous homologous fragments from DNA sequences of terrestrial poxviruses demonstrate the genetic relatedness of these virus fragments. The DNA polymerase and DNA topoisomerase phylograms indicate that the CPV-1 and CPV-2 viruses group together and form a unique branch,

separate from the known poxvirus genera (Figures 3-23 and 3-24). The SSLPV also forms its own branch in both the DNA polymerase and DNA topoisomerase phylogragms (Figures 3-23 and 3-24). The phylogenetic tree constructed using the major envelope protein gene fragments amplified form pinniped parapoxviruses and numerous homologous fragments from DNA sequences of terrestrial poxviruses demonstrates the placement of the HSPPV, SSPPV and SSPPV gene fragments into the branch including other terrestrial parapox viruses (Figure 3-25).

Virus Isolation

All attempts to isolate poxviruses from pinniped and cetacean fresh and frozen skin lesions were unsuccessful.

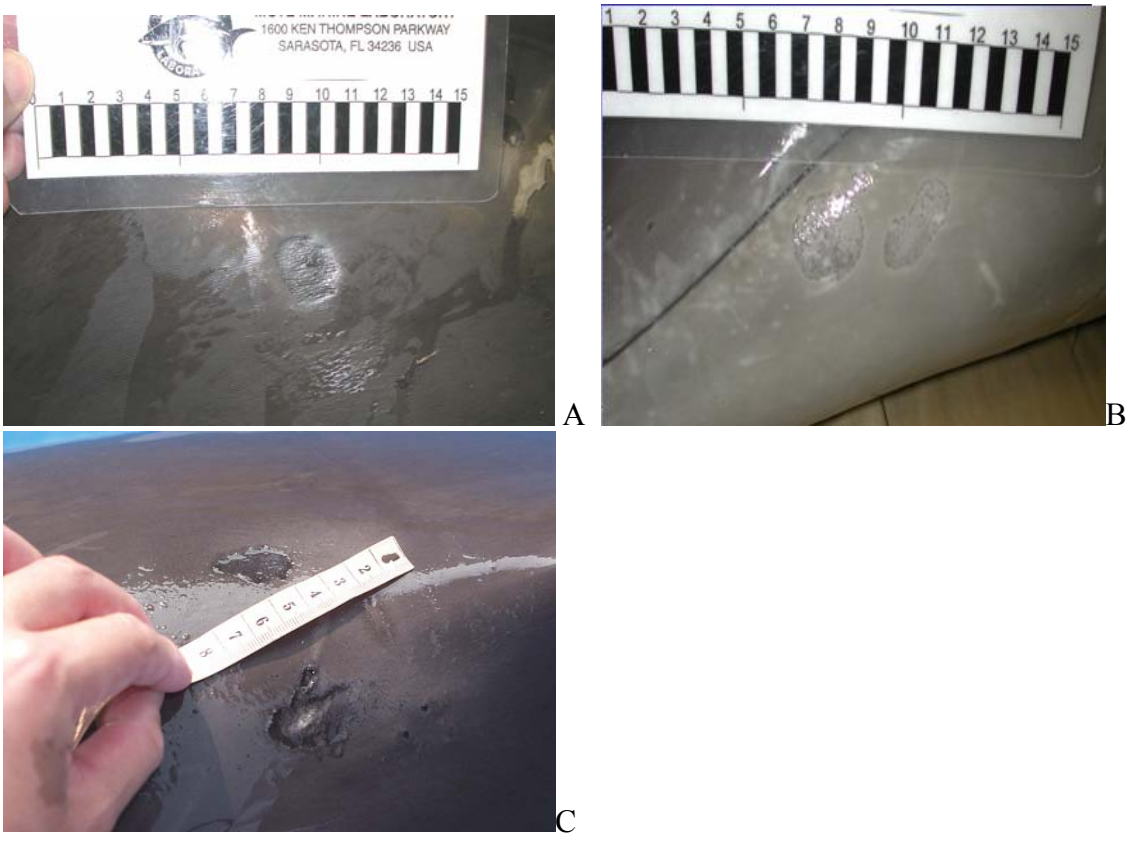


Figure 3-1.Typical "tattoo" lesions of cetaceans. A and B) Skin lesions of a rough-toothed dolphin (Steno bredanenesis). Photos taken by Dr. Charles Manire. C) Skin lesions of a bottlenose dolphin (Tursiops aduncus) from a Hong Kong aquarium.

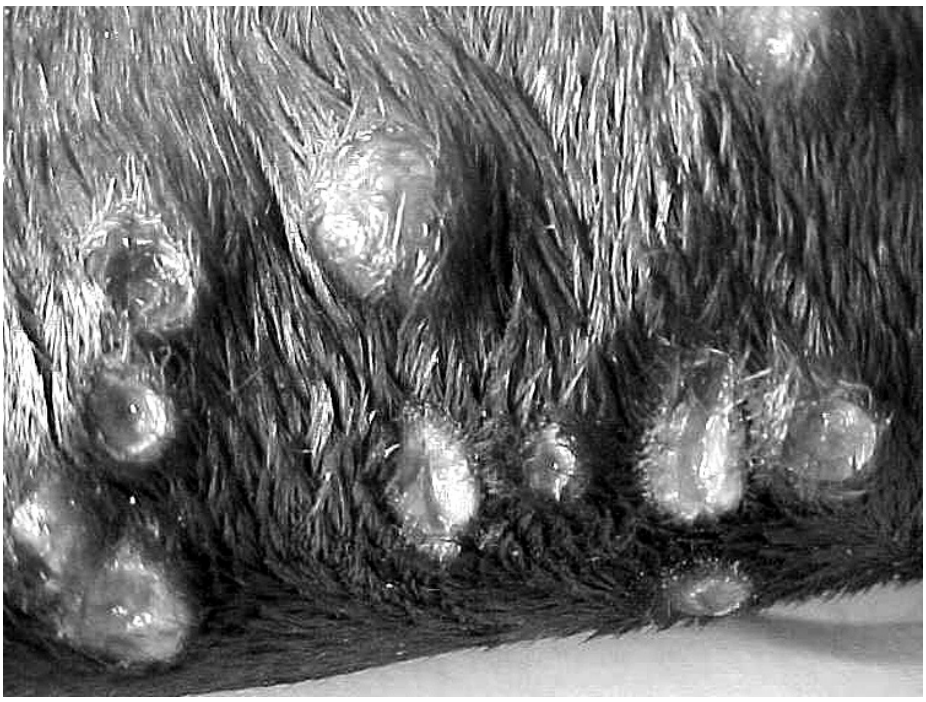


Figure 3-2 . Gross appearance of pox lesions associated with a poxvirus in a Steller sea lion (Eumetopias jubatus). Approximately 1 cm diameter raised smooth, hairless, often umbilicated, nodules were scattered across the body. Photo supplied by Dr. Kathy Burek.

Figure 3-3. Cutaneous pox lesions in a spotted seal (Phoca largha) associated with spotted seal parapoxvirus. Photo supplied by Dr. Kathy Burek.

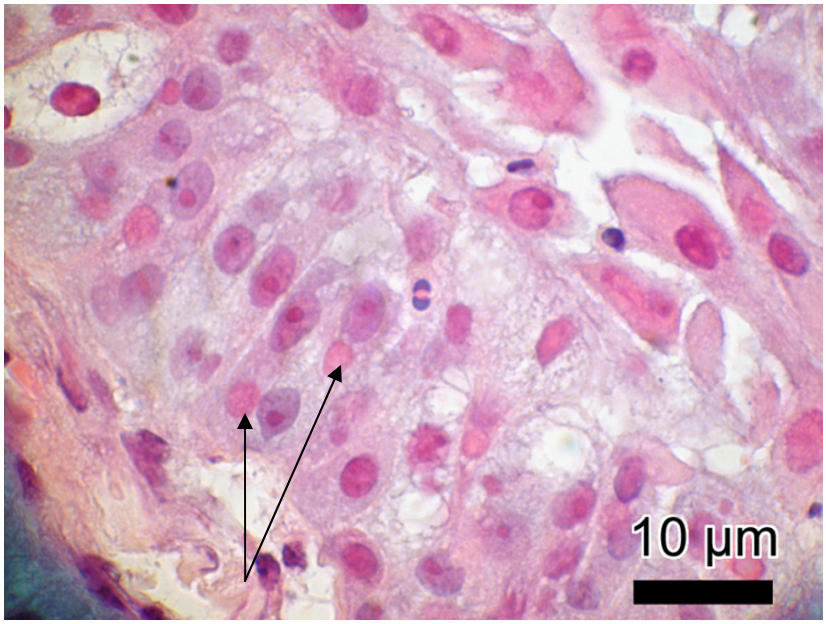


Figure 3-4 . Histopathologic appearance of cutaneous lesions associated wtih Steller sea lion poxvirus, showing epithelial cells containing acidophilic intracytoplasmic inclusion bodies (arrow). Slide supplied by Dr. Kathy Burek.

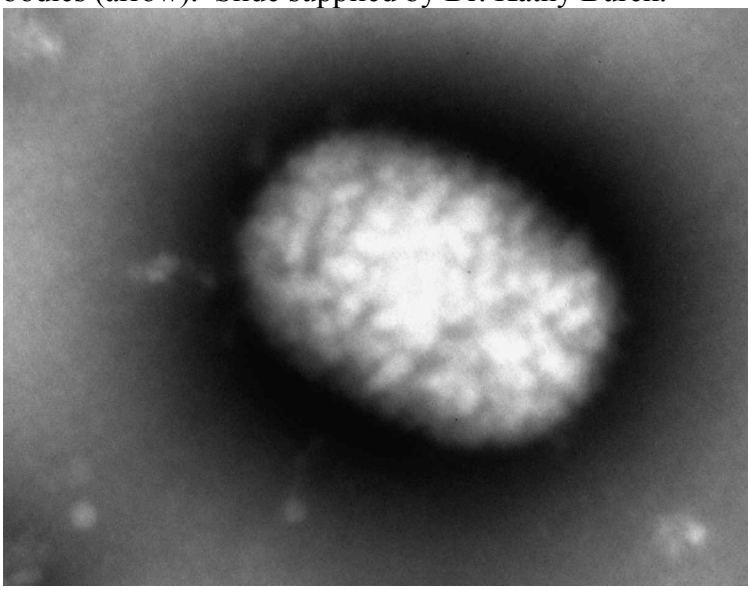
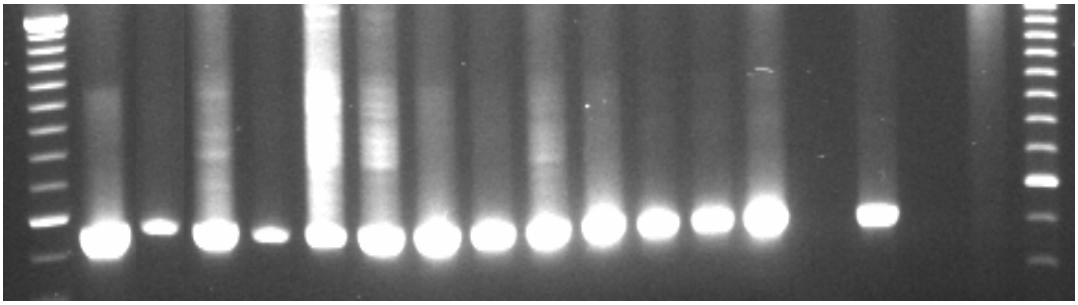
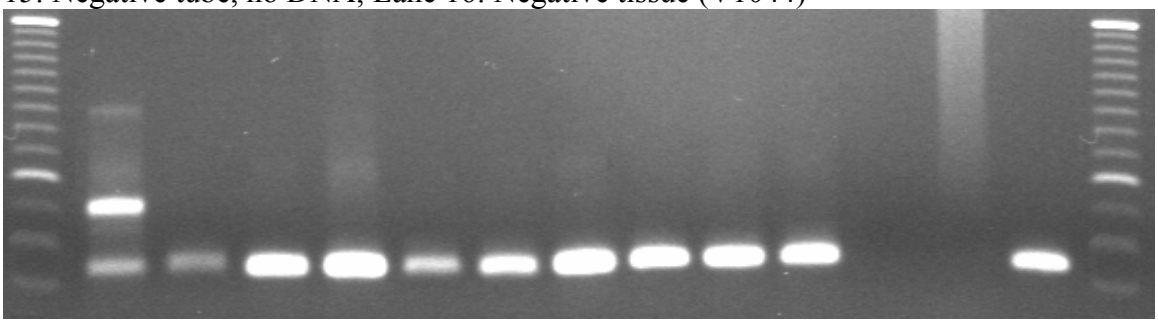


Figure 3-5. Negatively stained poxvirus particle from cutaneous lesion of SSL observed by electron microscopy. The 'skew' pattern of orthopoxviruses is evident as opposed to the 'criss-cross' pattern of parapoxviruses. Photo supplied by Mr. Woody Fraser.



M.M. 1 2 3 4 5 6 7 8 9 10 11 12 13 -- 14 15 16 M.M.

Figure 3-6. Agarose gel electrophoresis of PCR amplified 543-546-bp fragments of the DNA polymerase gene of cetacean and Steller sea lion poxviruses using primers CR 421 and CR 422. M.M.: 1KB Plus Molecular Ladder; Lane 1: Rough-toothed dolphin (V365); Lane 2: Rough-toothed dolphin (GW010006D); Lane 3: Bottlenose dolphin (R127); Lane 4: Bottlenose dolphin (V466); Lane 5: Bottlenose dolphin (V550); Lane 6: Bottlenose dolphin (V551); Lane 7: Bottlenose dolphin (MML0203); Lane 8: Bottlenose dolphin (OK04091932); Lane 9: Bottlenose dolphin (CMA0108); Lane 10: Bowhead whale (98KK3); Lane 11: Steller sea lion (SSL2001-279); Lane 12: Steller sea lion (SSL2000-105); Lane 13: Steller sea lion (SSL2005-546); Lane 14: Positive control, MDPV; Lane 15: Negative tube, no DNA; Lane 16: Negative tissue (V1044)



M.M. 1 2 3 4 5 6 7 8 9 10 11 12 13 M.M.

Figure 3-7. Agarose gel electrophoresis of PCR amplified 344-bp fragments of the DNA topoisomerase gene of cetacean and Steller sea lion poxviruses using primers CR 432 and CR 433. M.M.: 1KB Plus Molecular Ladder; Lane 1: Rough-toothed dolphin (V365); Lane 2: Rough-toothed dolphin (GW010006D); Lane 3: Bottlenose dolphin (R127); Lane 4: Bottlenose dolphin (MML0203); Lane 5: Bottlenose dolphin (OK04091932); Lane 6: Bottlenose dolphin (CMA0108); Lane 7: Bowhead whale (98KK3); Lane 8: Steller sea lion (SSL2001-279); Lane 9: Steller sea lion (SSL2000-105); Lane 10: Steller sea lion (SSL2005-546); Lane 11: Negative water, no DNA; Lane 12: Negative tissue (V1044); Lane 13: Positive control, MDPV DNA

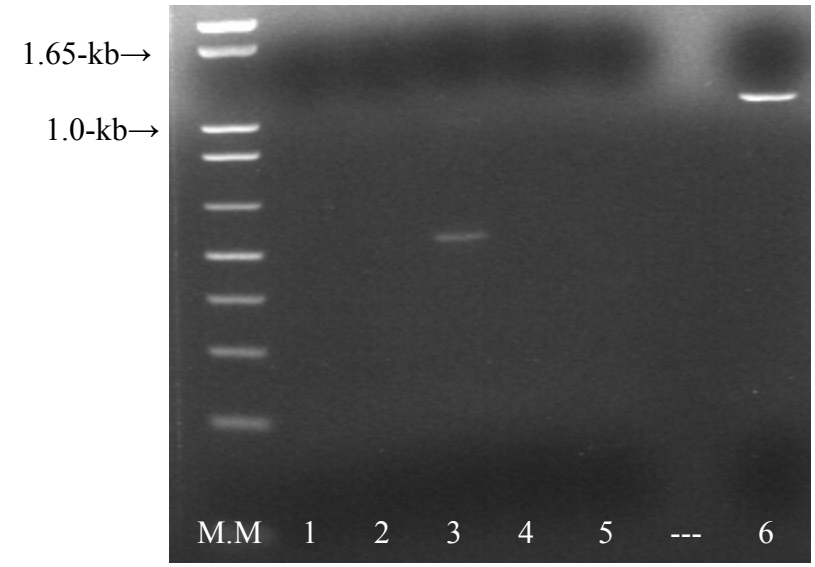


Figure 3-8. Agarose gel electrophoreses of PCR amplified fragments of the HA gene of orthopoxviruses using primers CR 619 and CR620 targeting a 1183-bp. M.M.: 1 KB Plus Molecular Ladder; Lane 1: Bottlenose dolphin CPV-1 (V1546); Lane 2: Bowhead whale CPV-2 (V730); Lane 3: Steller sea lion poxvirus (R227); Lane 4: Negative tissue (V1044); Lane 5: Negative water, no DNA; Lane 6: Vaccinia virus DNA

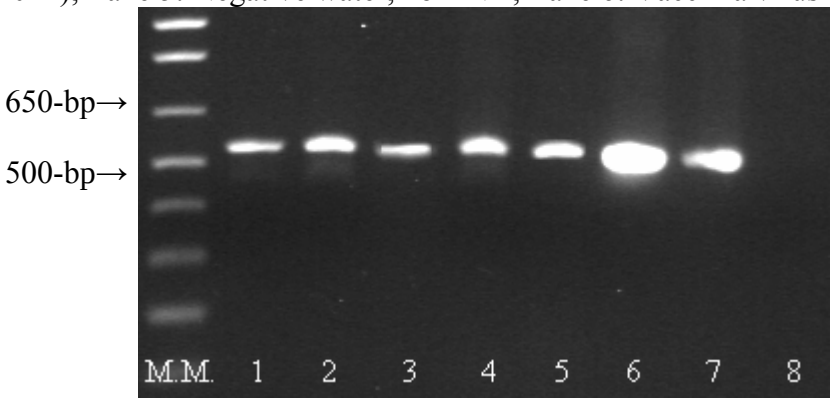


Figure 3-9. Agarose gel electrophoresis demonstrating the PCR amplification of 536-bp parapox DNA polymerase gene fragments from lesions of different pinniped species. M.M.: 1 KB Plus molecular ladder; Lane 1: Steller sea lion (SSL2003-450); Lane 2: Steller sea lion (SSL2003-451); Lane 3: Steller sea lion (SSL2004-495); Lane 4: Harbor seal (MMSC 03021); Lane 5: Spotted seal (DIO-136-03); Lane 6: Spotted seal (DIO-119-03); Lane 7: positive control, pseudocowpox virus DNA; Lane 8: negative control, no DNA template.

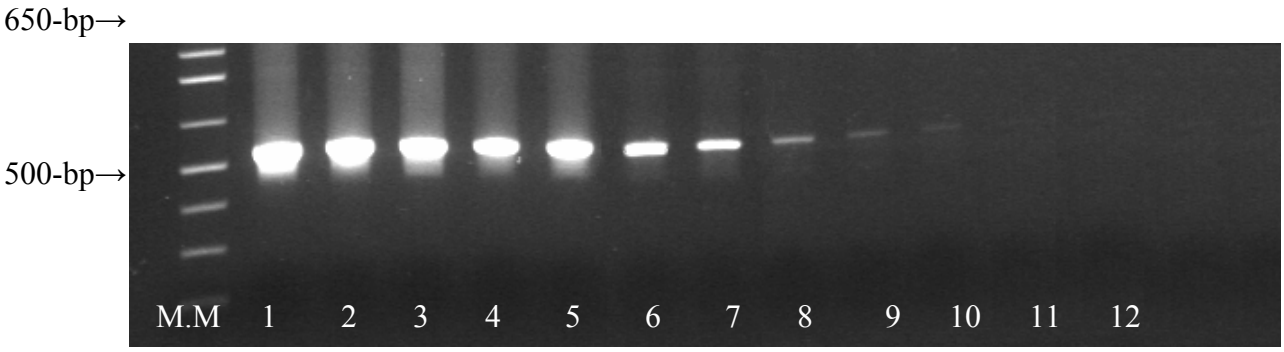


Figure 3-10. Agarose gel electrophoresis demonstrating the PCR sensitivity assay for primers CR540 and CR541 targeting the parapox DNA polymerase gene. pCR-II topo 2.1 plasmid vector containing the 536-bp fragment amplified from the Spotted seal (DIO-136-03) parapoxvirus DNA in 10-fold serial dilutions. M.M: 1 Kb plus ladder. Lane 1: 100 ng; Lane 2: 10 ng; Lane 3: 1.0 ng; Lane 4: 100 pg; Lane 5: 10 pg; Lane 6: 1.0 pg; Lane 7: 100 fg, Lane 8: 10 fg; Lane 9: 1.0 fg; Lane 10: 0.1 fg; Lane 11: 0.01 fg; Lane 12: 0.001; Lane 13: negative control, no DNA.

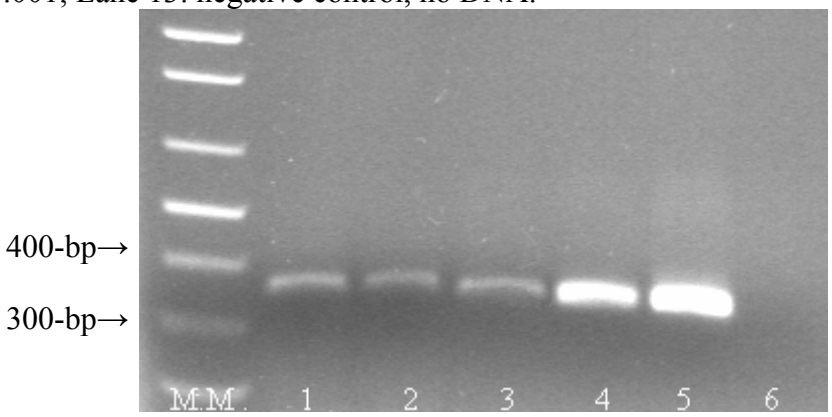


Figure 3-11. Agarose gel electrophoresis demonstrating the PCR amplification of 350-bp parapox DNA topoisomerase I gene fragments from lesions of Steller sea lions (Eumetopias jubatus) and harbor seals (Phoca vitulina). M.M.: 1KB Plus molecular ladder; Lane 1: Steller sea lion (SSL2003-450); Lane 2: Steller sea lion (SSL2003-451); Lane 3: Steller sea lion (SSL2004-495); Lane 4: Harbor seal (MMSC 03021); Lane 5: positive control, pseudocowpox virus DNA; Lane 6: negative control, no DNA template.

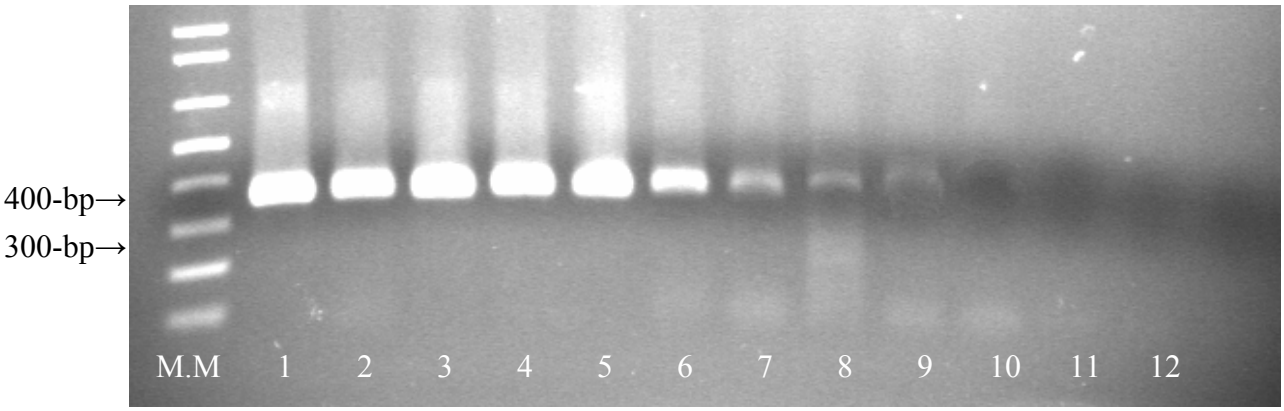


Figure 3-12. Agarose gel electrophoresis demonstrating the PCR sensitivity assay for primers CR550 and CR551 targeting the Steller sea lion (SSL2003-451) parapoxvirus DNA topoisomerase gene. pCR-II topo 2.1 plasmid vector containing the 350-bp fragment in 10-fold serial dilutions. M.M: 1 Kb plus ladder. Lane 1: 100 ng; Lane 2: 10 ng; Lane 3: 1.0 ng; Lane 4: 100 pg; Lane 5: 10 pg; Lane 6: 1.0 pg; Lane 7: 100 fg, Lane 8: 10 fg; Lane 9: 1.0 fg; Lane 10: 0.1 fg; Lane 11: 0.01 fg; Lane 12: 0.001; Lane 13: negative control, no DNA.

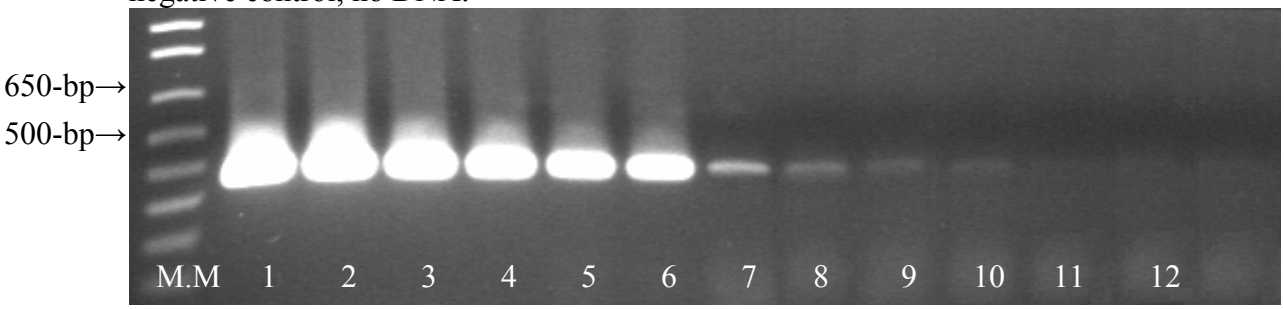


Figure 3-13. Agarose gel electrophoresis demonstrating the PCR sensitivity assay for primers CR557 and CR558 targeting the harbor seal DNA topoisomerase gene. pCR-II topo 2.1 plasmid vector containing the 350-bp parapoxvirus DNA topoisomerase fragment in 10-fold serial dilutions. M.M: 1 Kb plus ladder. Lane 1: 100 ng; Lane 2: 10 ng; Lane 3: 1.0 ng; Lane 4: 100 pg; Lane 5: 10 pg; Lane 6: 1.0 pg; Lane 7: 100 fg, Lane 8: 10 fg; Lane 9: 1.0 fg; Lane 10: 0.1 fg; Lane 11: 0.01 fg; Lane 12: 0.001; Lane 13: negative control, no DNA.

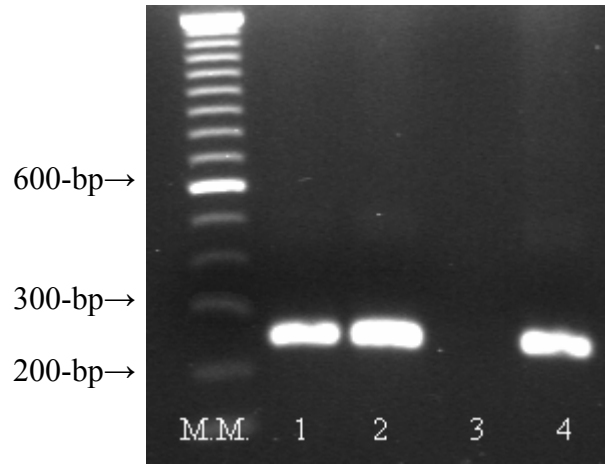


Figure 3-14. Agarose gel electrophoresis demonstrating the PCR amplification of 252-bp parapox virus DNA topoisomerase gene fragment from lesions of spotted seals (Phoca largha). M.M.: 100-bp molecular ladder; Lane 1: Spotted seal (DIO-136-03); Lane 2: Spotted seal (DIO-119-03); Lane 3: negative control, no DNA template; Lane 4: positive control, Steller sea lion (SSL2003-451).

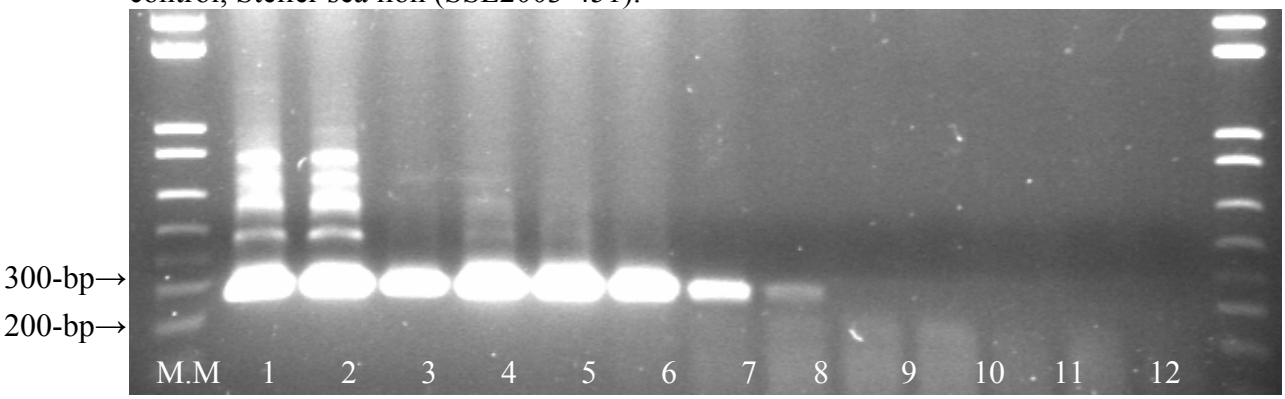


Figure 3-15. Agarose gel electrophoresis demonstrating the PCR sensitivity assay for primers CR570 and CR571 targeting the parapoxvirus DNA topoisomerase gene. pCR-II topo 2.1 plasmid vector containing the 252-bp fragment amplified from the Spotted seal (DIO-136-03) parapoxvirus DNA in 10-fold serial dilutions. M.M: 1 Kb plus ladder. Lane 1: 10 ng; Lane 2: 1.0 ng; Lane 3: 100pg; Lane 4: 10 pg; Lane 5: 1.0 pg; Lane 6: 100 fg; Lane 7: 10 fg, Lane 8: 1.0 fg; Lane 9: 0.1 fg; Lane 10: 0.01 fg; Lane 11: 0.001 fg; Lane 12: negative control; Lane 13: M.M .

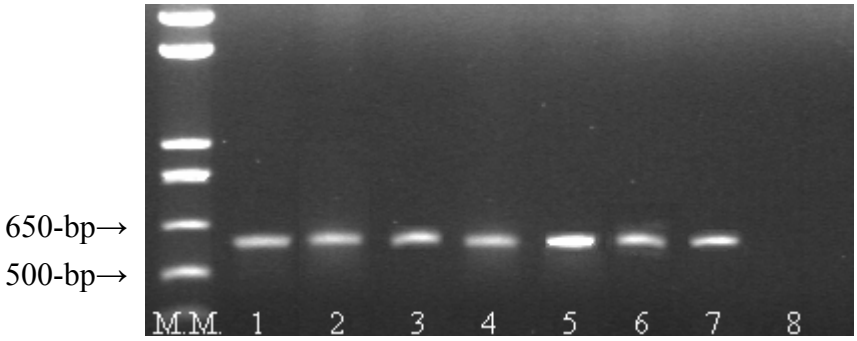


Figure 3-16. Agarose gel electrophoresis demonstrating the PCR amplification of 594-bp parapox major envelope protein gene fragment from lesions of different pinniped species. M.M.: 1 KB Plus molecular ladder; Lane 1: Steller sea lion (SSL2003-450); Lane 2: Steller sea lion (SSL2003-451); Lane 3: Steller sea lion (SSL2004-495); Lane 4: Harbor seal (MMSC 03021); Lane 5: Spotted seal (DIO-136-03); Lane 6: Spotted seal (DIO-119-03); Lane 7: positive control, pseudocowpox virus DNA; Lane 8: negative control, no DNA template.

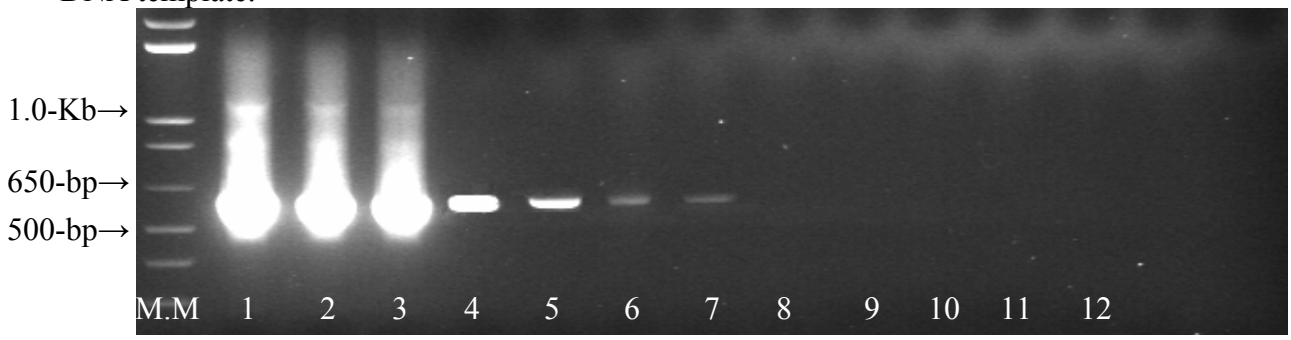


Figure 3-17. Agarose gel electrophoresis demonstrating the PCR sensitivity assay for primers CR339 and CR340 targeting the parapox major envelope protein gene. pCR-II topo 2.1 plasmid vector containing the 596-bp fragment amplified from the Harbor seal (MMSC03021) parapoxvirus DNA in 10-fold serial dilutions. M.M: 1 Kb plus ladder. Lane 1: 100 ng; Lane 2: 10 ng; Lane 3: 1.0 ng; Lane 4: 100 pg; Lane 5: 10 pg; Lane 6: 1.0 pg; Lane 7: 100 fg, Lane 8: 10 fg; Lane 9: 1.0 fg; Lane 10: 0.1 fg; Lane 11: 0.01 fg; Lane 12: 0.001; Lane 13: negative control, no DNA.

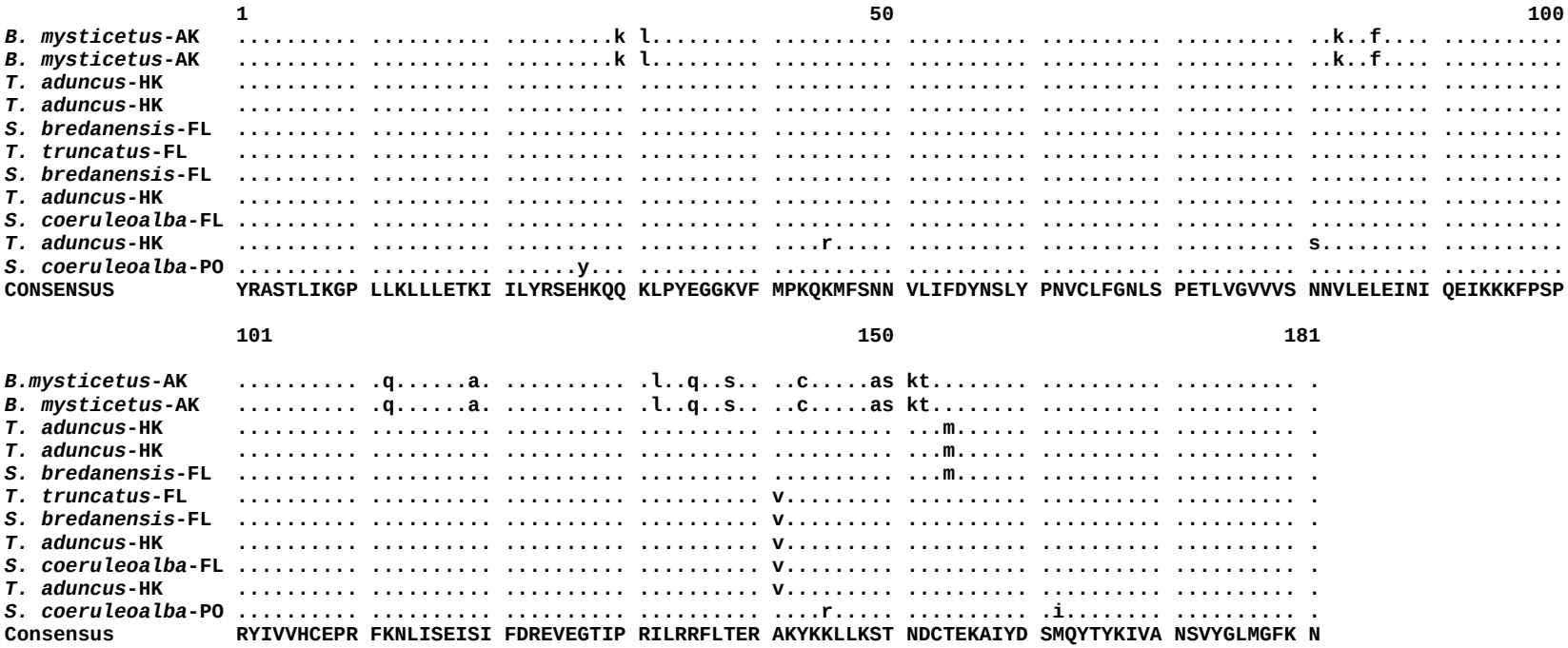


Figure 3-18. Multiple alignment of the amino acid sequences deduced from the nucleotide sequences of the DNA polymerase gene fragments of poxviruses identified in cutaneous lesions of cetaceans. AK = Alaska; FL = Florida; HK = Hong Kong; PO= Portugal.

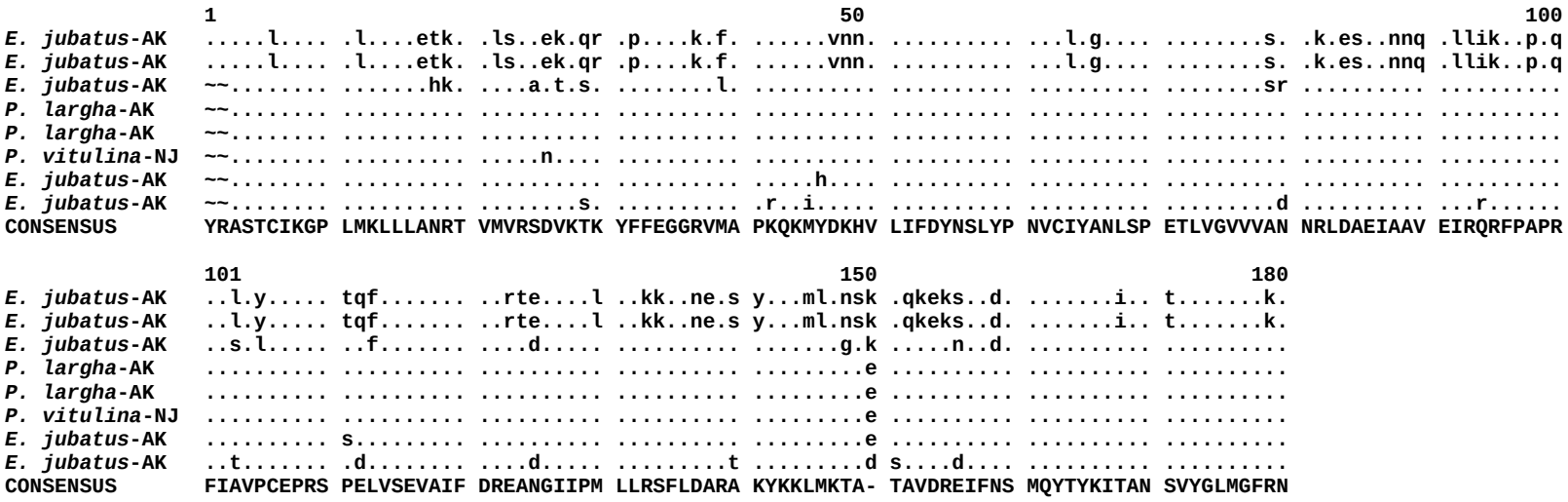


Figure 3-19. Multiple alignment of the amino acid sequences deduced from the nucleotide sequences of the DNA polymerase gene fragment of poxviruses identified in cutaneous lesions of pinnipeds. AK = Alaska; NJ = New Jersey.

```
                  1                                                          60
B. mysticetus-AK  .......... .......... .......... .dik....i. ..i....... .....yn.r.
B. mysticetus-AK  .......... .......... .......... .dik....i. ..i....... .....yn.r.
T. truncatus-FL   .......... .......... .......... .......... .......... ..........
S. bredanensis-FL .......... .......... .......... .......... .......... ..........
T. aduncus-HK     .......... .......... .......... .......... .......... ..........
S. coeruleoalba-FL .......... .......... .......... .......... .......... ..........
S. bredanensis-FL .......... .......... .......n.. ..f....... ........a. ..........
S. coeruleoalba-PO .......... .......... .......... ..i....... ..m....... ..........
CONSENSUS         METSFFIRTG KLRYLKENNT VGLLTLKSKH LTLTKDKLTI SFTGKDKVSH EFVIRRYDKL

                  61                                                        114
B. mysticetus-AK  .....k.a.. .d........ ...r...... nq...h.... .......... ....
B. mysticetus-AK  .....k.a.. .d........ ...r...... nq...h.... .......... ....
T. truncatus-FL   .......... .......... .......... .......... .......... ....
S. bredanensis-FL .......... .......... .......... .......... .......... ....
T. aduncus-HK     .......... .......... .......... .......... .......... ....
S. coeruleoalba-FL .......... .......... .......... .......... .......... ....
S. bredanensis-FL .......... .......... .......... .q........ .......... ....
S. coeruleoalba-PO .....k.... .d.......r .......... k......... .......... ....
CONSENSUS         YKPLIRLSKN KESECFLFDK LNENIIYKLI RPFGIRIKDL RTYGVNYTFL YNFW
```

Figure 3-20. Multiple alignment of the amino acid sequences deduced from the nucleotide. Sequences of the DNA topoisomerase gene fragments of poxviruses identified in cutaneous lesions of cetaceans.
AK = Alaska; FL = Florida; HK = Hong Kong; PO = Portugal.

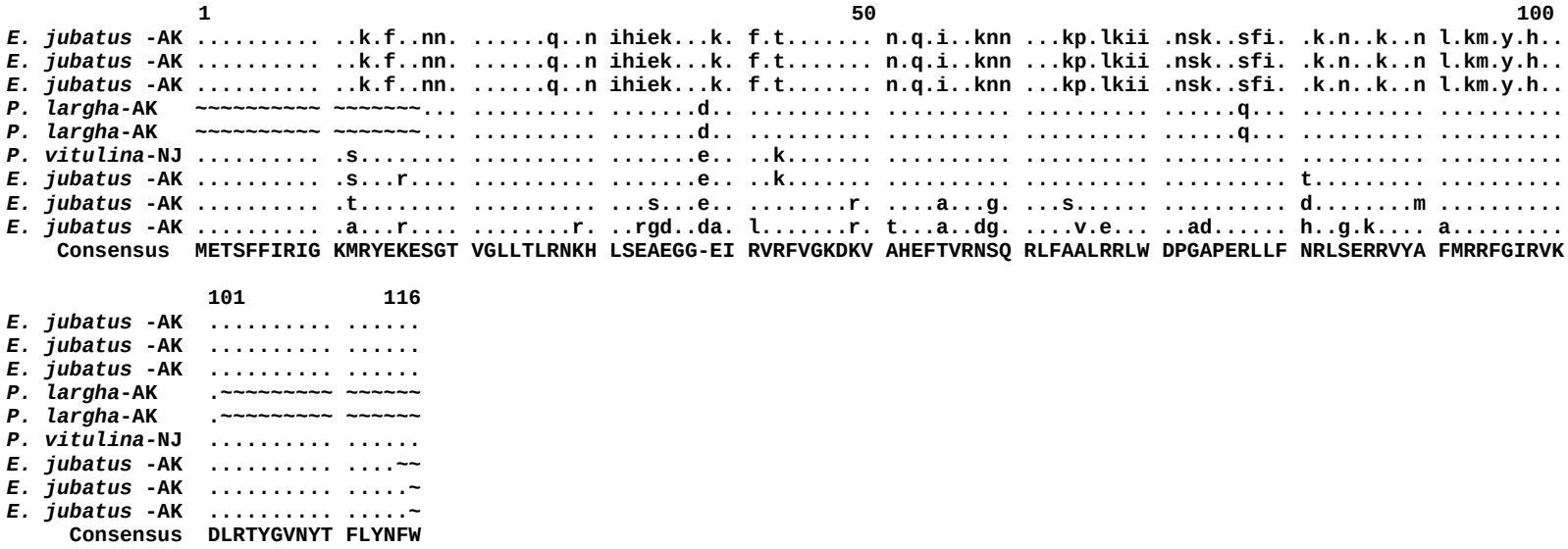


Figure 3-21. Multiple alignment of the amino acid sequences deduced from the nucleotide sequences of the DNA topoisomerase gene fragment of poxviruses and parapoxviruses identified in cutaneous lesions of pinnipeds. AK = Alaska; NJ = New Jersey.

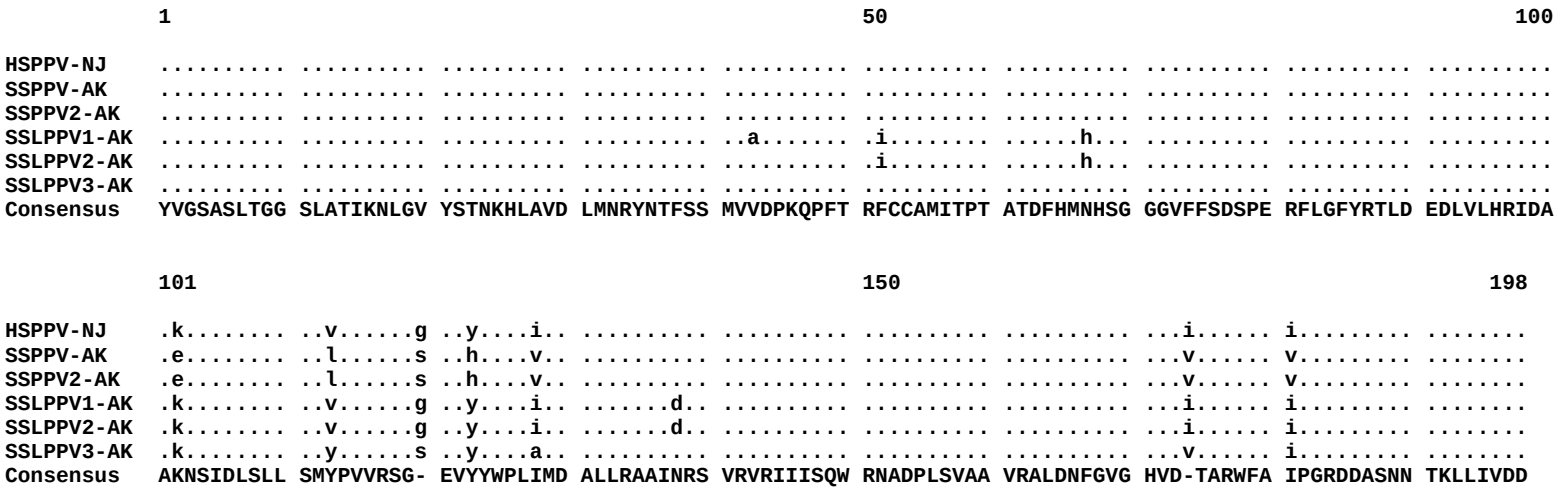
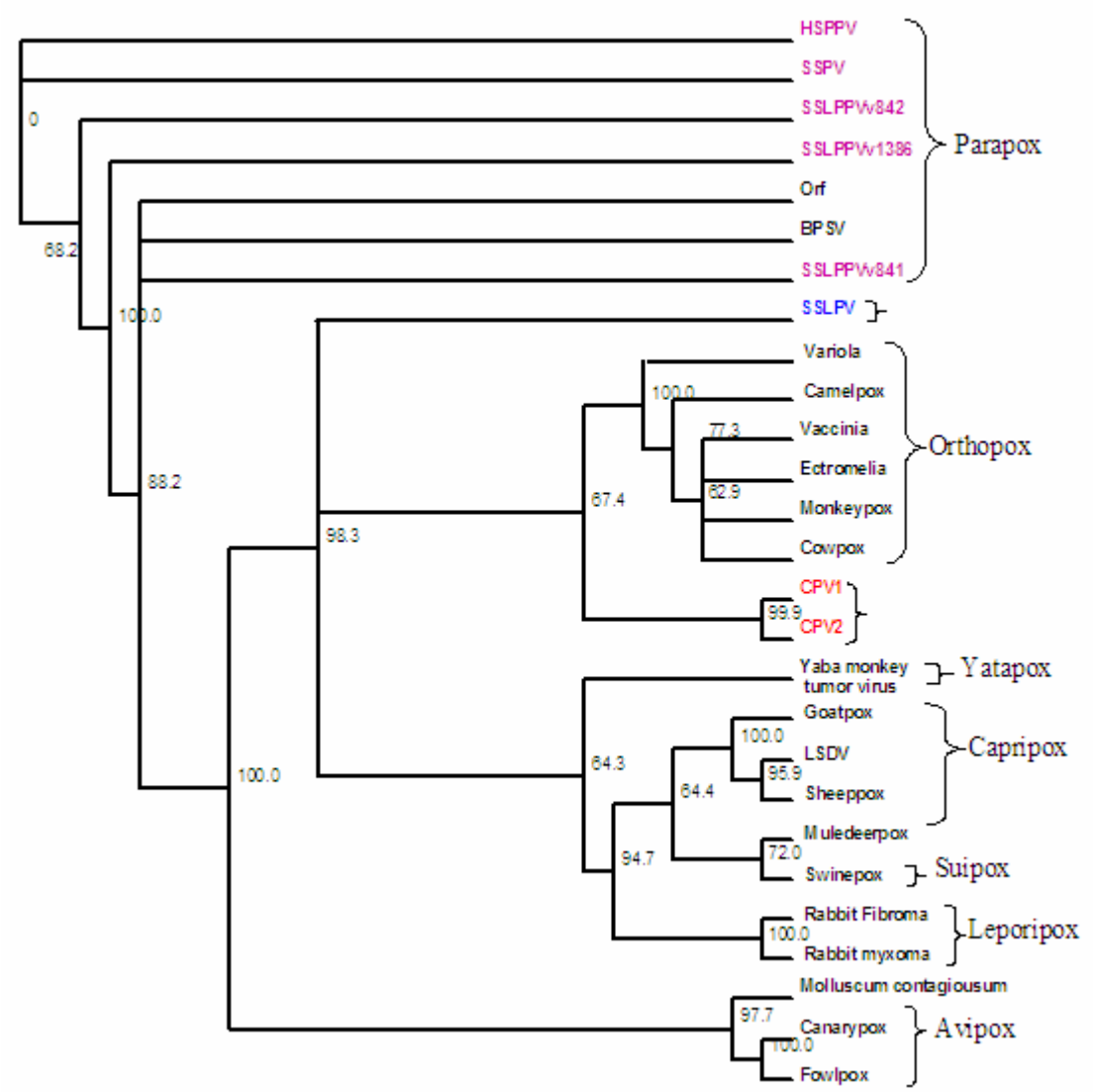
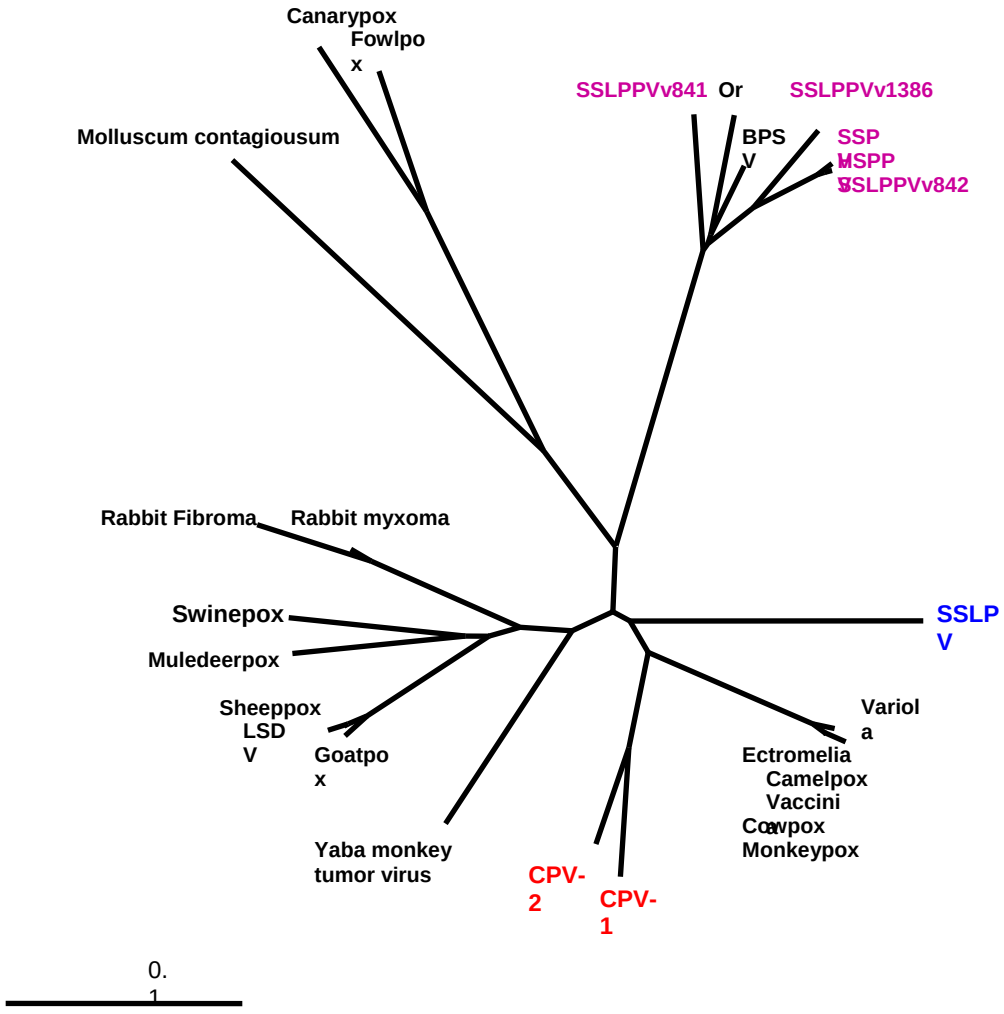


Figure 3-22. Multiple alignment of the partial amino acid sequences predicted from the major envelope protein gene fragment of parapoxviruses identified in cutaneous lesions of pinnipeds. AK = Alaska; NJ = New Jersey.



A.

Figure 3-23. Neighbor-Joining phylogenetic tree of the deduced amino acid sequences of the DNA polymerase gene fragments from members of the Chordopoxvirinae subfamily of poxviruses. The tree generated by Clustal X slow and accurate function using Gonnet 250 residue weight table, gap penalty of 11 and gap length penalty of 0.2. A) Format is a rectangular cladogram where the numbers represent the percent confidence of 1000 bootstrap replications. B) Radial format showing a .1 divergence scale representing 0.1 substitutions per site.



B.

Figure 3-23. Continued.

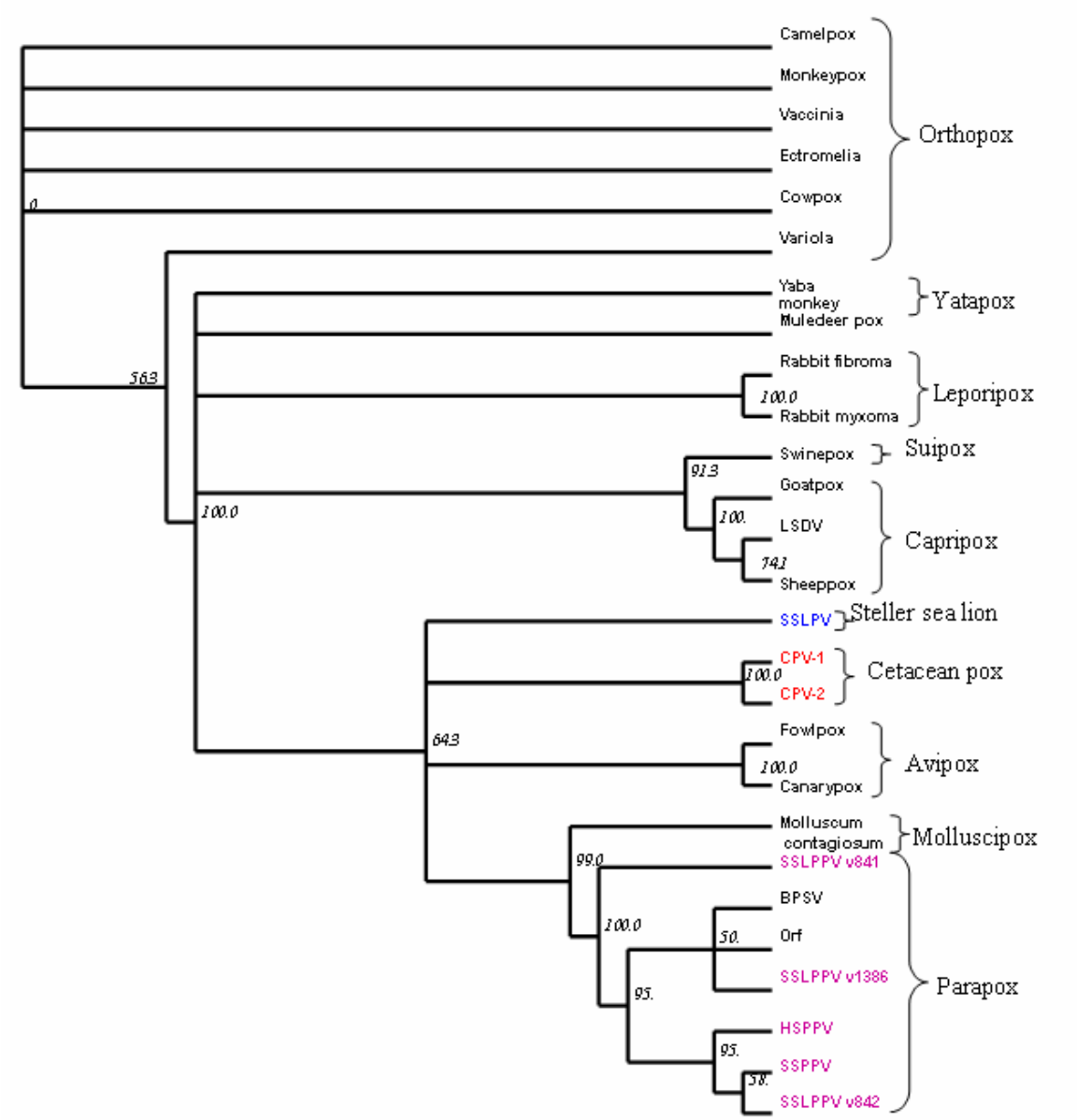


Figure 3-24. Neighbor-Joining phylogenetic tree of the deduced amino acid sequences of the DNA topoisomerase gene fragments from members of the Chordopoxvirinae subfamily of poxviruses. The tree was generated by Clustal X slow and accurate function using Gonnet 250 residue weight table, gap penalty of 11 and gap length penalty of 0.2. A) Format is a rectangular cladogram where the numbers represent the percent confidence of 1000 bootstrap replications. B) Radial format showing a .1 divergence scale representing 0.1 substitutions per site.

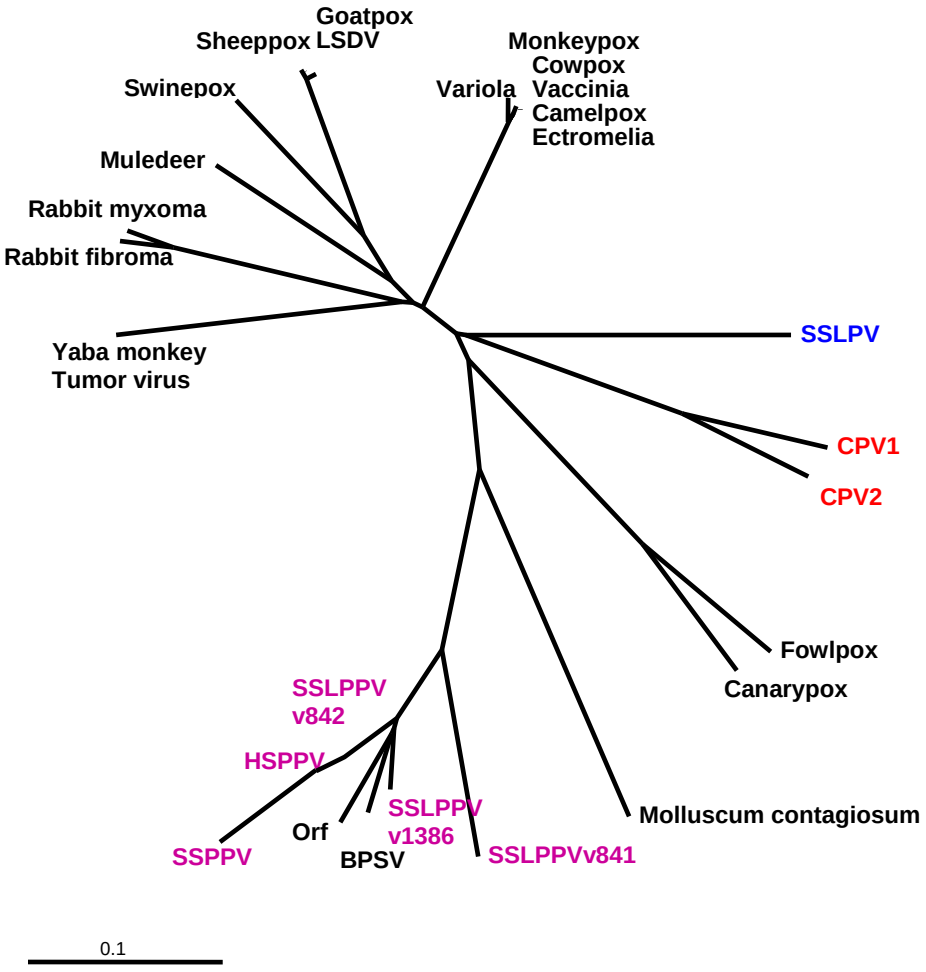


Figure 3-24. Continued.

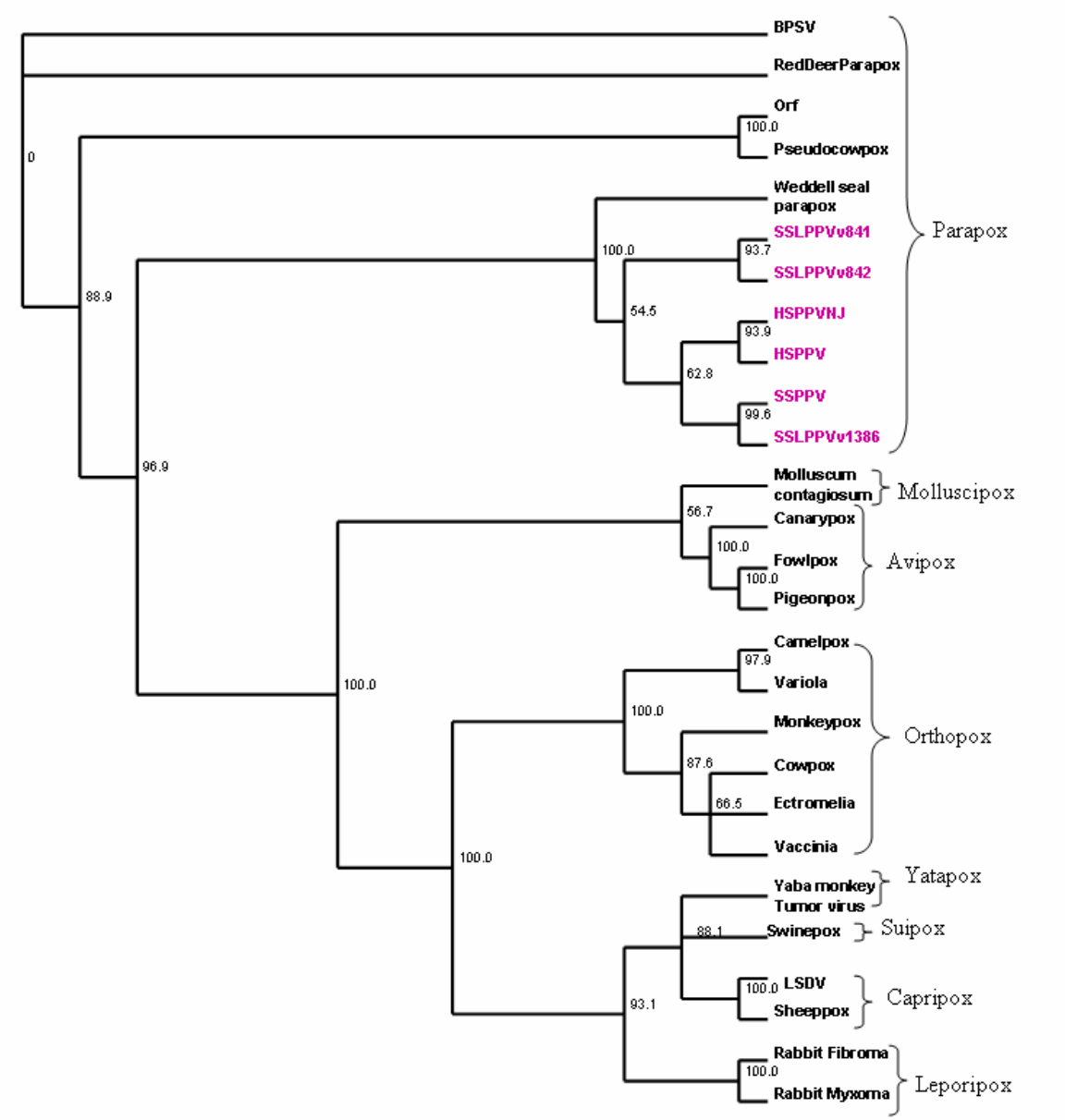


Figure 3-25 Neighbor-Joining phylogenetic tree of the deduced amino acid sequences of the Major envelope protein gene fragments from members of the Chordopoxvirinae subfamily of poxviruses. The tree was generated by Clustal X slow and accurate function using Gonnet 250 residue weight table, gap penalty of 11 and gap length penalty of 0.2. A) Format is a rectangular cladogram where the numbers represent the percent confidence of 1000 bootstrap replications. B) Radial format showing a .1 divergence scale representing 0.1 substitutions per site

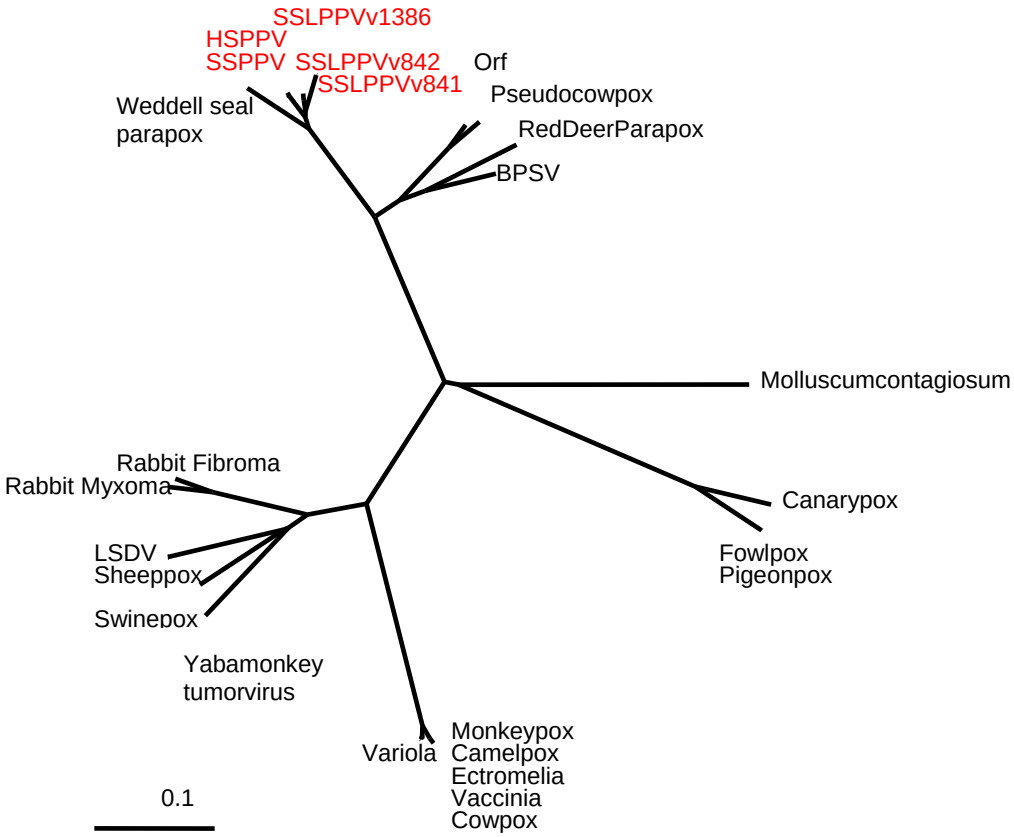


Figure 3-25. Continued.

Table 3-1. Pair-wise comparisons of the nucleotide sequences obtained from the DNA polymerase gene fragments of the cetacean poxvirus 1 (CPV-1) and cetacean poxvirus 2 (CPV-2) samples. Values correspond to percent identity between two nucleotide sequences.

	CPV-1 AJ28	**CPV-1 Wiki**	**CPV-1 R174**	**CPV-1 R164**	**CPV-1 V365**	**CPV-1 V1546**	**CPV-1 V466**	**CPV-1 V550**	**CPV-1 V551**	**CPV-2 V729**
CPV-1 AJ28	100.0									
CPV-1 Wiki	100.0	100.0								
CPV-1 R174	100.0	100.0	100.0							
CPV-1 R164	100.0	100.0	100.0	100.0						
CPV-1 V365	96.3	96.3	96.3	96.3	100.0					
CPV-1 V1546	93.0	93.0	93.0	93.0	91.9	100.0				
CPV-1 V466	99.1	99.1	99.1	99.1	95.8	92.5	100.0			
CPV-1 V550	96.2	96.2	96.2	96.2	99.5	92.1	95.6	100.0		
CPV-1 V551	96.3	96.3	96.3	96.3	99.6	92.3	95.8	99.8	100.0	
CPV-2 V729	84.4	84.41	84.4	84.4	84.4	84.1	83.5	84.6	84.4	100.0

Table 3-2. Pair-wise comparisons of the amino acid sequences deduced from the nucleotide sequences of DNA polymerase gene fragments of the cetacean poxvirus 1 (CPV-1) and cetacean poxvirus 2 (CPV-2) samples. Values correspond to percent identity between two amino acid sequences.

	CPV-1 AJ28	**CPV-1 Wiki**	**CPV-1 R174**	**CPV-1 R164**	**CPV-1 V365**	**CPV-1 V1546**	**CPV-1 V466**	**CPV-1 V550**	**CPV-1 V551**	**CPV-2 V729**
CPV-1 AJ28	100.0									
CPV-1 Wiki	100.0	100.0								
CPV-1 R174	100.0	100.0	100.0							
CPV-1 R164	100.0	100.0	100.0	100.0						
CPV-1 V365	98.9	98.9	98.9	98.9	100.0					
CPV-1 V1546	97.2	97.2	97.2	97.2	97.2	100.0				
CPV-1 V466	98.9	98.9	98.9	98.9	97.8	96.1	100.0			
CPV-1 V550	98.9	98.9	98.9	98.9	100.0	97.2	97.8	100.0		
CPV-1 V551	98.9	98.9	98.9	98.9	100.0	97.2	97.8	100.0	100.0	
CPV-2 V729	89.0	89.0	89.0	89.0	89.0	87.9	87.8	89.0	89.0	100.0

Table 3-3. Pair-wise comparisons of the amino acid sequences deduced from the nucleotide sequences of the DNA polymerase gene fragments of the cetacean poxvirus 1 (CPV-1) and cetacean poxvirus 2 (CPV-2) samples. Values correspond to percent similarity between two amino acid sequences.

	CPV-1 AJ28	**CPV-1 Wiki**	**CPV-1 R174**	**CPV-1 R164**	**CPV-1 V365**	**CPV-1 V1546**	**CPV-1 V466**	**CPV-1 V550**	**CPV-1 V551**	**CPV-2 V729**
CPV-1 AJ28	100.0									
CPV-1 Wiki	100.0	100.0								
CPV-1 R174	100.0	100.0	100.0							
CPV-1 R164	100.0	100.0	100.0	100.0						
CPV-1 V365	98.9	98.9	98.9	98.9	100.0					
CPV-1 V1546	98.9	98.9	98.9	98.9	98.9	100.0				
CPV-1 V466	99.4	99.4	99.4	99.4	98.3	98.3	100.0			
CPV-1 V550	98.9	98.9	98.9	98.9	100.0	98.9	98.3	100.0		
CVP-1 V551	98.9	98.9	98.9	98.9	100.0	98.9	98.3	100.0	100.0	
CPV-2 V729	92.3	92.3	92.3	92.3	92.3	92.3	91.7	92.3	92.3	100.0

Table 3-4. Pair-wise comparisons of the nucleotide sequences obtained from the DNA topoisomerase gene fragments of cetacean poxvirus 1 (CPV-1) and cetacean poxvirus 2 (CPV-2) samples. Values correspond to percent identity between two nucleotide sequences.

	CPV-1 AJ28	**CPV-1 Wiki**	**CPV-1 R174**	**CPV-1 R164**	**CPV-1 V365**	**CPV-1 V1546**	**CPV-2 V729**
CPV-1 AJ28	100.0						
CPV-1 Wiki	100.0	100.0					
CPV-1 R174	100.0	100.0	100.0				
CPV-1 R164	100.0	100.0	100.0	100.0			
CPV-1 V365	93.6	93.6	93.6	93.6	100.0		
CPV-1 V1546	92.4	92.4	92.4	92.4	89.8	100.0	
CPV-2 V729	84.3	84.3	84.3	84.3	84.9	86.0	100.0

Table 3-5. Pair-wise comparisons of the amino acid sequences deduced from the nucleotide sequences of the DNA topoisomerase gene fragments of cetacean poxvirus 1 (CPV-1) and cetacean poxvirus 2 (CPV-2) samples. Values correspond to percent identity between two amino acid sequences.

	CPV-1 AJ28	**CPV-1 Wiki**	**CPV-1 R174**	**CPV-1 R164**	**CPV-1 V365**	**CPV-1 V1546**	**CPV-2 V729**
CPV-1 AJ28	100.0						
CPV-1 Wiki	100.0	100.0					
CPV-1 R174	100.0	100.0	100.0				
CPV-1 R164	100.0	100.0	100.0	100.0			
CPV-1 V365	96.5	96.5	96.5	96.5	100.0		
CPV-1 V1546	94.7	94.7	94.7	94.7	92.1	100.0	
CPV-2 V729	85.1	85.1	85.1	85.1	84.2	86.8	100.0

Table 3-6. Pair-wise comparisons of the amino acid sequences deduced from the nucleotide sequences of the DNA topoisomerase gene fragments of cetacean poxvirus 1 (CPV-1) and cetacean poxvirus 2 (CPV-2) samples. Values correspond to percent similarity between two amino acid sequences.

	CPV-1 AJ28	**CPV-1 Wiki**	**CPV-1 R174**	**CPV-1 R164**	**CPV-1 V365**	**CPV-1 V1546**	**CPV-2 V729**
CPV-1 AJ28	100.0						
CPV-1 Wiki	100.0	100.0					
CPV-1 R174	100.0	100.0	100.0				
CPV-1 R164	100.0	100.0	100.0	100.0			
CPV-1 V365	96.5	96.5	96.5	96.5	100.0		
CPV-1 V1546	99.1	99.1	99.1	99.1	95.6	100.0	
CPV-2 V729	90.4	90.4	90.4	90.4	88.6	90.4	100.0

Table 3-7. Pair-wise comparisons of the nucleotide sequences of the DNA polymerase gene fragments of poxviruses of various genera within the Chordopoxvirinae subfamily of viruses. Values correspond to percent identity between two nucleotide sequences.

	CPV-1	**CPV-2**	**SSLPV**	**SSLPPV V841**	**SSLPPV V842**	**SSLPPV V1386**	**SSPPV V688**	**HSPPV V465**
Cetaceanpox-1	100.0	84.4	75.7	59.1	57.6	56.9	58.0	57.8
Cetaceanpox-2	84.4	100.0	77.0	59.0	55.2	54.3	56.7	56.5
Steller sealionpox	75.7	77.0	100.0	58.8	55.0	53.2	55.2	55.0
Camelpox	71.6	75.5	72.0	59.7	57.5	56.5	57.6	57.3
Cowpox	71.8	75.9	72.0	60.3	57.5	57.8	58.2	58.0
Monkeypox	72.6	76.1	72.4	60.1	57.5	57.1	57.6	57.3
Vaccinia	72.7	76.2	72.6	60.1	57.8	57.3	58.0	57.6
Ectromelia	72.0	76.1	71.6	60.1	58.8	58.0	59.0	58.6
Variola	71.6	74.8	71.8	60.8	58.4	57.5	58.6	58.2
Lumpy skin disease	74.0	74.4	74.0	61.2	56.7	56.9	56.7	56.3
Sheeppox	73.3	74.8	73.3	61.2	57.1	56.7	57.1	56.7
Goatpox	74.0	75.5	75.0	60.3	56.2	56.2	56.2	55.8
Muledeerpox	73.6	75.2	76.6	59.9	55.7	55.3	55.9	55.5
Swinepox	75.0	76.8	75.5	60.6	55.6	56.3	55.4	55.4
Rabbit fibroma	67.8	67.4	68.9	60.1	59.1	61.9	59.3	59.7
Rabbit myxoma	69.2	68.3	69.6	62.1	61.4	64.7	61.9	61.9
Yaba monkeypox	68.5	70.4	70.4	60.1	59.9	59.5	60.3	60.1
Orf	53.4	53.2	49.8	77.2	82.5	83.0	83.0	82.8
Bovine pap stom	53.4	51.6	50.6	77.3	81.3	83.8	81.9	81.5
Canarypox	40.4	41.5	40.6	36.1	36.9	36.8	32.2	32.3
Fowlpox	40.1	40.6	66.3	36.6	36.0	36.0	35.9	36.4
Molluscum conagiosum	50.5	51.6	48.3	57.3	60.4	62.7	60.4	60.4
Harbor sealparapox	57.8	56.5	55.0	79.7	98.3	84.0	99.1	100.0
Spotted sealparapox	58.0	56.7	55.2	79.5	98.7	84.3	100.0	99.1
Steller sealionparapox V841	59.1	59.0	58.8	100.0	78.9	77.1	79.5	79.7
Steller sealionparapox V842	57.6	55.2	55.0	78.9	100.0	84.3	98.7	98.3
Steller sealionparapox V1386	56.9	54.3	53.2	77.1	84.3	100.0	84.3	84.0

Table3-8. Pair-wise comparisons of the amino acid sequences deduced from the nucleotide sequence of the DNA polymerase gene fragments of poxviruses of various genera within the Chordopoxvirinae subfamily of viruses. Values correspond to percent identity between two amino acid sequences.

	CPV-1	**CPV-2**	**SSLPV**	**SSLPPV V841**	**SSLPPV V842**	**SSLPPV V1386**	**SSPPV V688**	**HSPPV V465**
Cetaceanpox-1	100.0	89.0	74.4	60.7	62.4	60.7	62.4	62.4
Cetaceanpox-2	89.0	100.0	77.8	62.9	62.4	60.1	62.4	62.4
Steller sealionpox	74.4	77.8	100.0	62.4	61.2	59.6	61.2	61.2
Camelpox	80.0	82.2	76.1	65.2	62.9	62.9	63.5	63.5
Cowpox	80.6	82.8	77.2	66.3	64.1	64.0	64.6	64.6
Monkeypox	80.6	82.8	77.2	66.3	64.1	64.0	64.6	64.6
Vaccinia	80.6	82.8	77.2	66.3	64.1	64.0	64.6	64.6
Ectromelia	80.6	82.8	77.2	66.3	64.1	64.0	64.6	64.6
Variola	79.4	81.7	77.2	66.3	64.1	64.0	64.6	64.6
Lumpy skin disease	75.0	74.4	73.9	60.7	62.9	61.8	62.9	62.9
Sheeppox	73.9	73.3	72.8	60.1	62.4	61.2	62.4	62.4
Goatpox	74.4	75.6	74.4	60.1	62.4	61.2	62.4	62.4
Muledeerpox	75.0	75.0	73.2	60.8	62.1	59.6	61.5	61.5
Swinepox	72.2	73.9	71.7	62.9	62.4	61.8	62.4	62.4
Rabbit fibroma	74.3	72.1	72.1	60.5	60.5	59.9	60.5	60.5
Rabbit myxoma	78.3	76.1	76.7	62.9	64.1	63.5	64.1	64.1
Yaba monkeypox	75.0	75.6	73.9	62.9	59.6	59.0	60.1	60.1
Orf	62.2	63.9	60.6	86.5	88.8	86.5	88.8	88.2
Bovine pap stom	65.6	65.0	64.4	87.1	88.2	87.6	89.3	88.8
Canarypox	56.7	59.4	59.4	50.0	49.4	50.0	49.4	49.4
Fowlpox	58.3	60.6	61.1	50.6	51.7	51.7	51.1	51.1
Molluscum contagiosum	56.1	57.2	56.1	56.7	56.7	59.0	56.7	56.7
Harbor sealparapox	62.4	62.4	61.2	87.6	98.3	91.2	99.4	100.0
Spotted sealparapox	62.4	62.4	61.2	88.2	98.9	92.1	100.0	99.4
Steller sealionparapox V841	60.7	62.9	62.4	100.0	87.1	85.4	88.2	87.6
Steller sealionparapox V842	62.4	62.4	61.2	87.1	100.0	91.0	98.9	98.3
Steller sealionparapox V1386	60.7	60.1	59.6	85.4	91.0	100.0	92.1	91.6

Table 3-9. Pair-wise comparisons of the amino acid sequences deduced from the nucleotide sequences of the DNA polymerase gene fragments of poxviruses of various genera within the Chordopoxvirinae subfamily of viruses. Values correspond to percent similarity between two amino acid sequences.

	CPV-1	**CPV-2**	**SSLPV**	**SSLPPV V841**	**SSLPPV V842**	**SSLPPV V1386**	**SSPPV V688**	**HSPPV V465**
Cetaceanpox-1	100.0	92.3	81.1	74.7	74.2	74.2	74.7	74.2
Cetaceanpox-2	92.3	100.0	82.8	76.4	75.3	74.8	75.8	75.3
Steller sealionpox	81.1	82.8	100.0	78.1	75.8	74.7	76.4	75.8
Camelpox	84.4	85.0	82.2	77.5	75.3	76.4	76.4	75.8
Cowpox	85.0	85.6	83.3	78.7	76.4	77.5	77.5	77.0
Monkeypox	85.0	85.6	83.3	78.6	76.4	77.5	77.5	77.0
Vaccinia	85.0	85.6	83.3	78.6	76.4	77.5	77.5	77.0
Ectromelia	85.0	85.6	83.3	78.7	76.4	77.5	77.5	77.0
Variola	85.0	85.6	83.3	78.7	76.4	77.5	77.5	77.0
Lumpy skin disease	80.6	81.1	80.6	74.7	75.8	75.8	76.4	75.8
Sheeppox	79.4	80.0	79.4	73.6	74.7	74.7	75.3	74.7
Goatpox	80.0	80.6	80.0	74.8	75.8	75.8	76.4	75.8
Muledeerpox	80.4	80.4	81.0	75.3	76.5	75.3	76.5	75.9
Swinepox	81.1	82.2	80.6	75.3	74.7	75.3	75.3	75.3
Rabbit fibroma	79.9	78.8	78.2	72.3	72.9	72.9	73.4	72.9
Rabbit myxoma	83.9	82.8	81.1	75.8	75.8	76.4	76.4	75.8
Yaba monkeypox	81.7	81.7	81.1	74.2	71.9	73.6	73.0	72.5
Orf	73.9	75.6	73.9	91.6	92.7	92.7	93.3	92.7
Bovine pap stom	77.8	78.3	78.9	93.3	92.1	92.1	92.7	92.1
Canarypox	69.4	70.6	68.9	68.0	65.7	66.3	66.3	66.3
Fowlpox	68.3	69.4	70.6	67.4	66.3	65.7	66.3	66.3
Molluscum contagiosum	75.0	74.4	72.2	71.9	70.2	70.3	70.8	70.8
Harbor sealparapox	74.2	75.3	75.8	91.6	98.9	94.9	99.4	100.0
Spotted sealparapox	74.7	75.8	76.4	92.1	99.4	95.5	100.0	99.4
Steller sealionparapox V841	74.7	76.4	78.1	100.0	91.6	91.0	92.1	91.6
Steller sealionparapox V842	74.2	75.3	75.8	91.6	100.0	94.9	99.4	98.9
Steller sealionparapox V1386	74.2	74.8	74.7	91.0	94.9	100.0	95.5	94.9

Table 3-10. Pair-wise comparisons of the nucleotide sequences of the DNA topoisomerase gene fragments of poxviruses of various genera within the Chordopoxvirinae subfamily of viruses. Values correspond to percent identity between two nucleotide sequences.

	CPV-1	**CPV-2**	**SSLPV**	**SSLPPV V841**	**SSLPPV V842**	**SSLPPV V1386**	**SSPPV V688**	**HSPPV V465**
Cetaceanpox-1	100.0	84.3	70.9	51.7	53.5	53.2	47.6	53.8
Cetaceanpox-2	84.3	100.0	72.1	59.0	52.3	54.3	56.7	56.5
Steller sealionpox	70.9	72.1	100.0	51.7	54.4	52.6	48.0	52.6
Camelpox	64.5	68.0	70.1	55.2	55.5	54.9	52.0	55.5
Cowpox	63.4	67.2	70.6	55.2	54.9	55.5	51.2	54.9
Monkeypox	64.2	68.0	71.2	55.2	54.9	54.4	51.2	54.9
Vaccinia	64.5	68.0	70.1	55.2	55.5	54.9	52.0	55.5
Ectromelia	64.5	68.0	70.9	55.2	55.5	54.9	52.0	55.5
Variola	64.8	68.3	71.2	54.9	55.2	54.7	51.6	55.2
Lumpy skin disease	69.5	68.3	72.1	52.0	53.2	51.5	45.5	51.7
Sheeppox	68.6	68.6	70.9	52.0	53.5	52.3	45.9	52.0
Goatpox	69.2	68.0	71.5	52.3	53.2	51.5	45.5	51.7
Muledeerpox	66.9	68.0	72.7	53.8	55.5	55.2	50.8	54.7
Swinepox	73.0	72.4	73.0	55.5	55.5	53.8	51.6	55.2
Rabbit fibroma	64.2	63.1	65.4	55.5	54.9	57.3	50.0	54.9
Rabbit myxoma	63.6	65.1	65.7	57.3	57.8	60.2	51.6	57.6
Yaba monkeypox	69.6	68.0	69.4	54.6	53.8	51.5	47.6	53.5
Orf	50.0	50.0	47.1	72.6	82.3	82.3	81.0	83.7
Bovine pap stom	51.2	50.6	48.5	72.3	81.4	81.4	80.2	83.4
Canarypox	62.8	64.0	68.0	52.3	52.9	54.9	50.0	52.3
Fowlpox	61.0	61.3	68.0	53.8	54.4	52.0	50.0	53.8
Molluscum contagiosum	50.3	50.3	49.1	61.6	68.0	65.4	65.4	68.3
Harbor sealparapox	53.8	56.5	52.6	75.5	96.0	85.0	95.6	100.0
Spotted sealparapox	47.6	56.7	48.0	71.9	93.7	84.3	100.0	95.6
Steller sealionparapox V841	51.7	59.0	51.7	100.0	74.4	70.6	71.9	75.5
Steller sealionparapox V842	53.5	52.3	54.4	74.4	100.0	82.3	93.7	96.0
Steller sealionparapox V1386	53.2	54.3	52.6	70.6	82.3	100.0	84.3	85.0

Table 3-11. Pair-wise comparisons of the amino acid sequences deduced from the nucleotide sequences of the DNA topoisomerase gene fragments of poxviruses of various genera within the Chordopoxvirinae subfamily of viruses. Values correspond to percent identity between two amino acid sequences.

	CPV-1	**CPV-2**	**SSLPV**	**SSLPPV V841**	**SSLPPV V842**	**SSLPPV V1386**	**SSPPV V688**	**HSPPV V465**
Cetaceanpox-1	100.0	85.1	60.5	56.1	57.0	57.9	46.4	57.9
Cetaceanpox-2	85.1	100.0	67.5	55.3	56.1	57.0	45.1	57.0
Steller sealionpox	60.5	82.8	100.0	57.0	57.0	57.0	46.3	58.9
Camelpox	64.9	66.7	69.3	57.9	63.2	62.3	57.3	64.0
Cowpox	64.9	66.7	69.3	57.9	63.2	62.3	57.3	64.0
Monkeypox	64.9	66.7	69.3	57.9	63.2	62.3	57.3	64.0
Vaccinia	64.6	66.8	69.0	57.5	62.8	61.9	57.3	63.7
Ectromelia	64.9	66.7	69.3	57.9	63.2	62.3	57.3	64.0
Variola	65.8	67.5	70.2	57.9	62.3	61.4	56.1	63.2
Lumpy skin disease	63.2	64.9	69.3	55.3	59.6	58.8	48.8	59.6
Sheeppox	63.2	64.9	69.3	55.3	59.6	58.8	48.8	59.6
Goatpox	63.2	65.8	69.3	55.3	59.7	58.8	48.8	59.6
Muledeerpox	62.3	64.0	69.3	57.0	61.4	60.5	52.4	62.3
Swinepox	64.9	65.8	67.5	58.8	59.6	59.6	52.4	60.5
Rabbit fibroma	62.3	62.3	64.9	58.8	57.8	57.0	48.8	58.8
Rabbit myxoma	64.0	62.3	64.9	58.7	58.8	57.0	48.8	58.8
Yaba monkeypox	63.2	60.5	62.3	57.9	59.6	57.9	50.0	60.5
Orf	55.3	54.4	53.5	79.1	89.7	91.4	85.7	87.9
Bovine pap stom	58.7	56.1	55.3	80.9	89.7	92.2	87.0	90.5
Canarypox	57.1	57.9	64.0	54.5	56.1	55.3	50.0	56.1
Fowlpox	55.3	51.8	61.4	52.6	56.1	55.3	46.3	56.1
Molluscum contagiosum	56.1	55.3	55.3	61.4	62.3	62.3	58.5	61.4
Harbor sealparapox	57.9	57.0	58.9	79.1	97.4	91.4	95.2	100.0
Spotted sealparapox	46.4	45.1	46.3	75.9	95.2	89.3	100.0	95.2
Steller sealionparapox V841	56.1	55.3	57.0	100.0	80.9	81.6	75.9	79.1
Steller sealionparapox V842	57.0	56.1	57.0	80.9	100.0	91.4	95.2	97.4
Steller sealionparapox V1386	57.9	57.0	57.0	81.6	91.4	100.0	89.3	91.4

Table 3-12. Pair-wise comparisons of the amino acid sequences deduced from the nucleotide sequences from the DNA topoisomerase gene fragments of poxviruses of various genera within the Chordopoxvirinae subfamily of viruses. Values correspond to percent similarity between two amino acid sequences.

	CPV-1	**CPV-2**	**SSLPV**	**SSLPPV V841**	**SSLPPV V842**	**SSLPPV V1386**	**SSPPV V688**	**HSPPV V465**
Cetaceanpox-1	100.0	90.4	75.4	67.5	66.7	67.5	57.3	65.8
Cetaceanpox-2	90.4	100.0	77.2	68.4	66.7	67.5	57.3	65.8
Steller sealionpox	75.4	82.8	100.0	65.8	70.2	70.2	62.2	71.1
Camelpox	75.4	77.2	76.3	71.1	73.7	73.7	70.7	74.6
Cowpox	75.4	77.2	76.3	71.1	73.7	73.7	70.7	74.6
Monkeypox	75.4	77.2	76.3	71.1	73.7	73.7	70.7	74.6
Vaccinia	75.2	77.0	76.1	70.8	73.5	73.5	70.7	74.3
Ectromelia	75.4	77.2	76.3	71.1	73.7	73.7	70.7	74.6
Variola	76.3	78.1	77.2	71.1	72.8	72.8	69.5	73.7
Lumpy skin	73.7	72.8	75.4	70.2	71.9	71.9	65.9	71.9
Sheeppox	73.7	72.8	75.4	70.2	71.9	71.9	65.9	71.9
Goatpox	73.7	72.8	75.4	71.1	71.9	71.9	48.8	71.9
Muledeerpox	72.8	72.8	75.4	68.4	70.2	69.3	64.6	71.1
Swinepox	75.4	74.5	79.8	73.7	73.7	72.8	68.3	74.6
Rabbit fibroma	71.9	74.6	72.8	71.1	68.4	66.7	59.8	68.4
Rabbit myxoma	71.9	74.6	72.8	71.1	68.4	66.7	59.8	68.4
Yaba monkeypox	70.0	70.2	73.7	69.3	70.2	69.3	63.4	71.1
Orf	67.5	67.5	70.2	86.1	93.1	94.8	90.5	93.1
Bovine pap stom	69.3	68.4	70.2	87.0	92.2	94.0	91.7	92.2
Canarypox	70.2	68.4	74.6	70.2	71.9	71.9	65.9	71.9
Fowlpox	69.3	65.8	73.7	68.4	70.2	70.2	63.4	70.2
Molluscum	68.4	67.5	70.2	69.3	71.9	71.9	69.5	71.1
Harbor sealparapox	65.8	75.3	75.8	86.1	99.1	94.0	98.8	100.0
Spotted sealparapox	57.3	75.8	76.4	80.7	97.6	91.7	100.0	98.8
Steller sealionparapox V841	67.5	76.4	78.1	100.0	86.1	86.0	80.7	86.1
Steller sealionparapox V842	66.7	75.3	75.8	86.1	100.0	94.0	97.6	99.1
Steller sealionparapox V1386	67.5	74.8	74.7	86.0	94.0	100.0	91.7	94.0

Table 3-13. Pair-wise comparisons of the nucleotide sequences obtained from the major envelope protein gene fragments of marine parapoxviruses within the Chordopoxvirinae subfamily of viruses.Values correspond to percent identity between two nucleotide sequences.

	SSLPPV V841	**SSLPPV V842**	**SSLPPV V1386**	**SSPPV V688**	**HSPPV V465**
Harbor sealparapox	93.4	93.3	93.3	93.1	100.0
Spotted sealparapox	91.6	91.4	95.8	100.0	93.1
Steller sealionparapox V841	100.0	99.8	94.8	91.6	93.4
Steller sealionparapox V842	99.8	100.0	94.9	91.4	93.3
Steller sealionparapox V1386	91.6	91.4	100.0	95.8	93.3

Table 3-14. Pair-wise comparisons of the amino acid sequences deduced from the nucleotide sequences from the major envelope protein gene fragments of marine parapoxviruses within the Chordopoxvirinae subfamily of viruses. Values correspond to percent identity between two amino acid sequences.

	SSLPPV V841	**SSLPPV V842**	**SSLPPV V1386**	**SSPPV V688**	**HSPPV V465**
Harbor sealparapox	97.0	96.5	96.0	96.0	100.0
Spotted sealparapox	93.9	93.4	100.0	100.0	96.0
Steller sealionparapox V841	100.0	99.5	93.9	93.9	97.0
Steller sealionparapox V842	99.5	100.0	93.4	96.5	96.5
Steller sealionparapox V1386	93.9	93.4	100.0	96.0	96.0

Table 3-15. Pair-wise comparisons of the amino acid sequences deduced from the nucleotide sequences of the major envelope protein gene fragments of marine parapoxviruses within the Chordopoxvirinae subfamily of viruses.Values correspond to percent similarity between two amino acid sequences.

	SSLPPV V841	**SSLPPV V842**	**SSLPPV V1386**	**SSPPV V688**	**HSPPV V465**
Harbor sealparapox	98.5	98.0	98.5	98.5	100.0
Spotted sealparapox	97.0	96.5	100.0	100.0	98.5
Steller sealionparapox V841	100.0	99.5	97.0	97.0	98.5
Steller sealionparapox V842	99.5	100.0	96.5	98.0	98.0
Steller sealionparapox V1386	97.0	96.5	100.0	98.5	98.5

CHAPTER 4
DISCUSSION

All of the cetacean poxvirus skin lesions examined in the present study conformed with the typical tattoo lesion appearance. Some lesions were over 2.0 cm in diameter, while some samples of lesions consisted of 8 mm diameter skin biopsies. We cannot make any conclusions regarding the specific stage of infection represented in each lesion, other than to observe that some lesions showed more definite hyperpigmentation of the skin, or more clearly defined edges surrounding the lesion. We did not find any association between cetacean lesion appearance and positive pox PCR results, or poxvirus DNA sequences obtained. Pinniped parapoxviruses are associated with skin lesions that resemble those reported for other terrestrial parapoxviruses such as those that are seen in orf, pseudocowpox, and bovine papular stomatitis, both histologically and in patterns of disease progression (Wilson et al., 1972; Hadlow et al., 1980; Hicks and Worthy, 1987). The prevalence of parapoxvirus infection in pinnipeds remains unreported; however, skin lesions associated with these infections are frequently encountered in both stranded pinnipeds brought into rehabilitation centers and in captive pinnipeds (Wilson et al., 1969; Wilson et al., 1972; Hadlow et al., 1980; Osterhaus et al., 1990; Simpson et al., 1994; Muller et al., 2003). Hicks and Worthy, (1987), reported that five of 11 recently weaned grey seal (Halochoerus grypus) pups collected for a nutritional study developed parapox lesions after 1 – 4 weeks in captivity. The appearance of these lesions in animals that appeared otherwise healthy at the time of collection, suggests that

while the pups may be exposed to the parapoxvirus in the wild population, the viral infection may be exacerbated under stressful conditions brought on in captivity. Pinniped skin lesions examined in this study were collected during Steller sea lion capture-release studies or by members of the Alaska Department of Fish and Game and the Marine Mammal Stranding Center in New Jersey. A gross distinction between the appearance of lesions associated with Steller sea lion poxvirus and lesions associated with pinniped parapoxvirus, could not be made.

While histopathology and electron microscopy are useful in confirming the presence of typical microscopic poxvirus lesions and in the visualization of viral particles in cetacean lesions (Flom and Houk, 1979; Geraci et al., 1979; Smith, 1983; Baker, 1992a,b; Van Bressem et al., 1993), they offer little information about the type of poxvirus involved. The primary objective of this study was to develop a diagnostic strategy based on extraction of total DNA from lesions, PCR assay using the extracted DNA as template, and sequencing of the amplified fragments, to detect and characterize poxviruses in cutaneous lesions of cetaceans and pinnipeds. The first step in creating a PCR protocol was to design oligonucleotide primers that would anneal to targeted genes in the template viral DNA present in cutaneous lesions. Problems encountered in designing primers to target cetacean and pinniped poxvirus genes, stemmed from the lack of any available genetic data pertaining to marine mammal poxviruses. Despite the absence of sound antigenic and molecular data, most previous work using electron microscopy on cetacean poxviruses has repeatedly implicated them as members of the orthopoxvirus genus. In addition, one study reported a mixed parapox and orthopox virus infection in a grey seal, based on the same techniques (Osterhaus et al., 1990). In the

present study, histopathologic examination of the two Steller sea lion skin lesions revealed a similar appearance to lesions of the northern fur seal and South American sea lions, characterized by dermal nodules of hyperplastic epithelial cells versus the raised plaque-like lesions described in harbor and grey seal lesions (Wilson and Poglayen-Neuwall, 1971; Wilson et al., 1972; Hicks and Worthy, 1987; Osterhaus et al., 1990, 1994). Electron microscopy performed on the two Steller sea lion skin lesions revealed the presence of poxvirus virions with morphologic characteristics consistent with published reports of orthopox viruses (Moss, 2001). However, sequencing of amplified fragments showed that most likely, these viruses are species specific poxviruses of Steller sea lions and not orthopoxviruses. Histopathologic and electron microscopic examination of the 10 positive cetacean skin lesions was not performed due to poor sample quality and in general, improper sample preservation.

Because of their high level of conservation within the Chordopoxvirinae, the DNA polymerase and DNA topoisomerase I genes were targeted for the design of oligonucleotide primers for PCR. Specifically, nucleotide sequences within regions of the open reading frame of these genes that were highly conserved within members of the Orthopox, Suipox, and Capripox genera were targeted with consensus primers to drive the amplification of approximately 543-bp in the case of the DNA polymerase gene and 344-bp for the DNA topoisomerase I gene. Sequences of the Orthopox, Suipox, and Capripox genera were obtained from the GenBank database and through the website of the National Center for Biotechnology Information (NCBI). The amount of sequence generated by fragments of the above sizes is usually sufficient to characterize viruses molecularly and assign them to proper virus genera and species, when derived from genes

that exhibit high levels of conservation (Ropp et al., 1995; Zanotto et al., 1996; Becher et al., 2002; McGeoch et al., 2000).

Most pinniped poxviruses have long been considered probable members of the Parapox genus. Inoshima et al., (2000), validated consensus primers that target a 596-bp gene fragment of the major envelope protein of parapoxviruses in ungulates. Using these primers in a PCR protocol, initially with suboptimal annealing temperatures, we were able to identify parapoxvirus positive samples from pinniped skin lesions and confirm the usefulness of the primers. A nucleotide alignment of orf and BPS viruses DNA sequences available in the NCBI was used to design PCR primers targeting a 536-bp fragment of the DNA polymerase gene and a 350-bp fragment of the DNA topoisomerase of parapoxviruses. These primers effectively amplified the respective genes of pinniped parapox viruses, confirming the diagnoses made using the major envelope protein gene primers. Our results expand the molecular diagnosis tools as applicable to parapoxvirus, and make possible a wider genetic analysis comprising two more genes. PCR protocols were developed using these primers at suboptimal annealing temperatures in order to maximize the chances of amplifying the cetacean and SSL poxvirus and pinniped poxvirus genes. Once each primer set was tested for reactivity using positive cetacean and/or pinniped poxvirus DNA, each protocol was optimized to produce a single amplicon, usually by raising the annealing temperature of PCR until the desired reactivity was obtained.

Positive samples were identified by the presence of a single amplicon of the expected size. DNA sequence was obtained by two methods; Firstly, cleaning of the PCR product followed by direct sequencing, and/or secondly, sequencing of the cloned

PCR product in the bacterial plasmid vector, PCR-Topo2.1. The first method was used when the amplified fragments were unique and allowed for the rapid diagnosis of poxvirus infection, and for verifying DNA sequences obtained from cloned products, when disparities between two or more cloned sequences were found. All samples that yielded positive results were later cloned, to obtain full sequences and to preserve valuable DNA products, as the amount of total DNA obtained from lesions was usually small and rapidly exhausted after multiple uses.

Ten cetacean skin lesions were found to contain amplifiable poxvirus DNA using the PCR protocols and DNA sequencing strategies described above. The identified positive samples represented two different groups of cetacean poxviruses, provisionally referred to as CPV-1 and CPV-2. Viruses in the CPV-1 corresponded to the poxvirus DNA polymerase and DNA topoisomerase sequences obtained from four species of dolphins while the CPV-2 virus corresponded to the DNA polymerase and DNA topoisomerase sequences of the bowhead whale (Balaena mysticetus) poxvirus. The same PCR protocols also amplified poxvirus DNA from two Steller sea lion skin lesions indicating the existence of a unique and most likely, species specific, Steller sea lion poxvirus (SSLPV). The three PCR assays for pinniped parapoxvirus allowed the identification of six positive skin lesion samples harvested from one harbor seal (HSPPV), two spotted seals (SSLPPV) and three Steller sea lions (SSLPPV). Although none of the Steller sea lions examined in this study showed evidence of a dual infection of both pox and parapoxviruses, we speculate that a dual infection could occur. Mammalian species that have been documented to be afflicted with multiple poxvirus species, belonging to different genera, include cattle,sheep and camels (Robinson and

Mercer, 1995; Inoshima et al., 2000; Moss, 2001). In cattle, infections with pseudocowpox virus, a member of the Parapox genus, and cowpox virus, a member of the Orthopox genus, have been observed (Pickup et al., 1982; Buller and Palumbo, 1991). In sheep, orf virus, of the Parapox genus, and sheeppox, of the Capripox genus, have been observed (Inoshima et al., 2000; Hosamani et al., 2004). In camels, camelpox, a notable member of the Orthopox genus, has been observed, as well as camel parapox virus (Robinson and Mercer, 1995; Gubser and Smith, 2002).

Nucleotide sequences and their deduced amino acid sequences obtained from all poxvirus positive samples were entered into the GenBank database and compared using pairwise and multiple alignment functions from the GCG Wisconsin Package. Pairwise comparisons were made between sequences obtained from each targeted gene of each of the cetacean and pinniped pox and parapox viruses, to sequences available in the GenBank and available in the NCBI database, representing several terrestrial poxviruses within the Chordopoxvirinae.

Considering first the DNA polymerase comparisons, the cetacean poxviruses share the highest homology among themselves, with a nucleotide identity of 84.4% (Table 3-7) and an amino acid identity of 89.0% (Table 3-8). The nucleotide identities described in Table 3-1 indicate that both CPV-1 and CPV-2 share the second closest identities to the SSL poxvirus, with identities of 75.7 and 77.0%. Following the SSL pox virus, CPV-1 and CPV-2 are most closely related to members of the Orthopox genus, with nucleotide identities ranging from 71.6 to 76.2% (Table 3-7). These viruses may have evolved from a common ancestor as species specific marine poxviruses, prior to the evolution of some of the terrestrial orthopoxviruses such as camelpox and some strains of the variola virus

(Afonso et al., 2002, Gubser and Smith, 2002). The cetacean poxviruses and SSL poxvirus shared the least homology with members of the Avipox and Parapox genera, with nucleotide identities below 53.2% (Table 3-7). These findings are supported by a previous phylogenetic study demonstrating the distant relationship of the orthopoxviruses to the avipoxviruses (Gubser et al., 2004). The pinniped parapox viruses shared highest nucleotide identities among themselves (Table 3-7). Notable are the nucleotide identities of 98.3 and 98.7% of one Steller sea lion, (V842), when compared to the harbor and spotted seal sequences (Table 3-7). The significance and interpretation of the identities are difficult to ascertain, as Steller sea lions and spotted seals inhabit northwest Pacific waters, while the harbor seal originated from northeast Atlantic waters. SSL V842 shared only 78.9 and 84.3% nucleotide identity to the other two SSL sequen ces. The nucleotide and amino acid identities between the harbor and spotted seals are above 98% (Table 3-7). These results suggest that the SSPPV, HSPPV and SSLPPV may have originated from a common ancestor and, diverged as they evolved with their host species. The pinniped parapoxviruses share the least homology to avipox viruses, as would be expected based on previous phylogenetic analysis of Chordopoxvirinae (Gubser et al., 2004). The amino acid identities represented in Table 3-8 show slightly different homologies. This is due to the nature of flexibility or degeneracy in the protein or amino acid code. In the nucleotide comparisons, each discrepancy between two nucleotide sequences is reported as a difference, whereas in translation to a protein sequence, a nucleotide substitution may be silent, causing no amino acid change, and thus no difference between the two sequences. Results from Table 3-8 indicate that the cetacean poxviruses are most homologous to each other, with the next closest homology being to

the orthopox viruses, followed by the SSL poxvirus. The Avipox and Parapox genera are consistently, the least homologous to the cetacean poxviruses. Protein identities of the pinniped parapoxviruses are highest to orf and BPSV, with the exception of the harbor and spotted seal poxviruses that share 99.4% identity to each other (Table 3-8). Variations in the 3 Steller sea lion DNA polymerase sequences are apparent in the protein identities with ranges from 85.4 to 91.0% (Table 3-8), suggesting the existence of more than one strain or type of Steller sea lion parapoxvirus. Comparisons among DNA polymerase protein similarities are reported in Table 3-9. Protein similarity comparisons offer a means to weigh the significance of observed amino acid differences. For example, the substitution of a basic amino acid for an acidic amino acid may cause a more significant functional change than a basic to basic amino acid substitution. The relevance of viewing the protein similarities of the gene sequences reported in Table 3-9 is simply to ascertain the significance of the amino acid differences indicated by the protein identities in Table 3-8 (Needleman and Wunsch, 1970). The homology patterns observed by looking at protein similarities agree with those reported for the protein identities, and warrant no further discussion.

Considering next, the DNA topoisomerase gene comparisons, the overall nucleotide identities are lower than those observed in the DNA polymerase comparisons, indicating a lesser degree of conservation in the DNA topoisomerase gene when compared to the DNA polymerase gene, within the Chordopoxvirinae (Table 3-10). The CPV-1 and CPV-2 fragments share 84.3% nucleotide identity with each other, followed by identities to swinepox virus of 72.4 to 73.0% (Table 3-10). The next closest homology is to SSL poxvirus followed by members of the Capripox genus (Table 3-10).

The difference in the pattern of homology between the DNA topoisomerase and DNA polymerase genes demonstrates the variability in gene evolution. Viral genes evolve at varying rates, depending on need to adapt to new host or environmental stresses (Upton et al., 2003; Gubser et al., 2004;). The pinniped parapoxviruses are consistently closest in homology to orf , BPSV and to each other (Table 3-10). Steller sea lion, V842, demonstrated a higher homology to the harbor and spotted seal sequences, than to the other (V841 and V1386) SSL sequences (Table 3-10), as seen in the DNA polymerase gene comparisons. The variance of the DNA topoisomerase amino acid identities from the DNA polymerase amino acid identities mimics these differences in the nucleotide identity tables. Pairwise comparisons of the amino acid identities indicate homologies of CPV-1 and CPV-2 to the Orthopox genus ranging from 64.6 to 67.5% (Table 3-11). CPV-1 shows only 60.5% amino acid identity to SSL poxvirus, while CPV-2 shows an amino acid identity to SSL poxvirus of 82.8% (Table 3-11). These different identities represent the differences in the evolutionary rates between the DNA polymerase and DNA topoisomerase genes examined in this study. However, these results indirectly confirm the differences between the cetacean poxviruses and indicate that CPV-2 is more closely related to the SSL poxvirus.

The major envelope protein gene (MEP) pairwise comparisons were made using exclusively the pinniped parapox gene sequences generated in this study. Attempts to amplify the MEP gene of cetacean and SSL poxviruses were unsuccessful, limiting the scope of the comparisons. The problems encountered in amplifying the MEP gene from the cetacean and SSL poxviruses stem from the degree of variation found between these novel poxviruses and other terrestrial poxviruses. The MEP gene of poxviruses is more

variable than the DNA polymerase and DNA topoisomerase genes (Upton et al., 2003). Primers designed based on the available MEP DNA sequences of other terrestrial poxviruses, most likely did not amplify the cetacean or SSL poxvirus MEP due to the greater degree of variation within the gene. The MEP gene comparisons demonstrate nucleotide and amino acid identities ranging from 91.4 to 99.8% (Table 3-13), and 93.4 to 99.5%, respectively (Table 3-14). The variance observed in the DNA polymerase and topoisomerase gene sequence comparisons were absent in the MEP comparisons and the homologies in the latter were more uniform. The MEP gene of poxviruses is typically more variable than those involved in DNA replication, as it is involved in host specificity, viral adhesion to the host cell, and possibly evasion of host immunity (Smith et al., 2002). Partial nucleotide and deduced amino acid sequences have been used to make a distinction between different species of parapoxvirus, such as orf , BPSV and pseudocowpox (Inoshima et al., 2000). Our results showed less variation in the pinniped parapoxvirus MEP gene fragments than the variation reported between homologous MEP gene fragments of orf, BPSV, and pseudocowpox (Inoshima et al., 2000; Becher et al., 2002; Delhon et al., 2004). These results may be due to the specific region of the gene amplified by the MEP PCR primers. Certain areas of the MEP gene are likely more conserved in DNA sequence, such as those encoding the hypdrophobic regions of the protein, found within the envelope lipid bilayer (Silverman, 2005). In addition, the poxviruses of marine mammals may not have succumbed to the same selective pressures encountered over hundreds of years by the terrestrial poxviruses, such as vaccination, husbandry and environmental conditions that stimulate genetic evolution and mutation in the viral genome. The high degree of conservation observed in the pinniped parapoxvirus

MEP sequences can be understood after considering the nature of these consensus PCR primers, designed to amplify the MEP gene fragment of all parapoxviruses.

Overall, it can be inferred, based on pairwise comparisons, that CPV-1, CPV-2 and SSLPV are most closely related to the orthopoxviruses, and that the pinniped parapoxviruses are most closely related to the known terrestrial parapoxviruses of ruminants. Phylogenetic trees were constructed using the deduced amino acid sequences, to further determine the genetic relatedness of the marine mammal poxviruses to known virus members of the Chordopoxvirinae.

The phylogenetic studies described in Upton et al. (2003), and Gubser et al. (2004), provided new insight into novel methods of analysis for uncharacterized poxviruses, such as those described in this thesis. In the present study, phylogenetic analysis was performed based on partial proteins of the DNA polymerase, DNA topoisomerase and major envelope protein genes of several members of the Chordopoxvirinae, including CPV-1, CPV-2, SSLPV, SSLPPV, SSPPV, and HSPPV (Figures 3-23A&B, 3-24A&B and 3-25A&B).

The bootstrapped cladogram and the radial divergence tree representing the DNA polymerase protein sequences indicate that the cetacean poxviruses form a distinct genus within the Chordopoxvirinae, separate from the Orthopox genus and from SSLPV, indicating a species specific poxvirus.. The SSLPV falls into a clad by itself, outside of the Orthopox lineage group. The pinniped parapoxviruses group, as expected, within the Parapox genus (Figure 3-23A). These results were reiterated in the divergence tree, revealing the ancestry of the DNA polymerase gene fragments within the Chordopoxvirinae. This tree clearly showed genetic divergence from the ancestor

branch, of SSLPV first, followed by the cetacean poxviruses, and finally, the differentiation of the orthopoxviruses (Figure 2-23B). Among the parapoxviruses, the SSLPPV sequences show three different points of divergence. SSLPPV(V841) diverged first, followed by orf and BPSV. SSLPPV(V1386), SSLPPV(V842), SSPPV, and HSPPV are branched together; however, SSLPPVv1386 diverges from the branch by itself. These results strongly suggest the existence of three different SSL parapoxviruses, supporting conclusions drawn from the pairwise comparison tables. These are the first sequences of SSL parapoxviruses ever obtained for the DNA polymerase gene.

Phylogenetic trees constructed based on partial proteins of the DNA topoisomerase gene indicate that CPV-1 and CPV-2 form a group separate from any other, as does SSLPV strongly suggesting that the viruses could be assigned to new genera within the Chordopoxvirinae subfamily of viruses (Figure 3-24A). All SSLPPVs, HSPPV, and SSPPV clad inside the parapox group. The radial divergence tree representing the DNA topoisomerase protein fragments differs from the DNA polymerase divergence tree (Figure 3-24B). The topoisomerase divergence tree shows the orthopox viruses as having a separate lineage from CPV-1, CPV2 and SSLPV, rather than the three groups diverging from a single branch. SSLPV is depicted closer to the Orthopox group, where as the DNA polymerase tree depicted the cetacean poxviruses closer to the Orthopox group. These results are further exemplification that the DNA topoisomerase I gene may have evolved at a different rate and direction than the DNA polymerase gene.

The final phylogenetic analyses performed were based on the partial protein sequences of the major envelope protein (MEP) (Figures 3-25A and 3-25B). Poxvirus MEP sequences were not obtained, and are, consequently, absent in the MEP

phylogenetic trees. Examination of the radial divergence tree indicates a closer relationship between the SSLPPV MEP fragments than those seen for the DNA polymerase and DNA topoisomerase genes (Figure 3-25B). These results agree with results obtained from the pairwise comparison tables. The radial tree (3-25B) also shows clear divergence of the pinniped parapox group, including a recently published sequence for the Weddel seal (Tryland et al., 2005), and from the other parapox species, namely orf, BPS, pseudocowpox, and parapox of red deer viruses.

Clear definitions for nucleotide and/or amino acid identity requirements necessary for the assignment of novel poxviruses to an appropriate genus are currently lacking. In the case of the orthopoxviruses, specifically variola, vaccinia and cowpox, the nucleotide identities are >90% (Goebel et al., 1990; Gubser et al., 2004). The newly identified cetacean poxviruses, CPV-1 and CPV-2, share only 84% nucleotide identity in the targeted regions of the DNA polymerase and DNA topoisomerase genes, and their nucleotide or amino acid identities with any members of the known poxvirus genera are even lower. Phylogenetic and evolutionary analysis of the DNA polymerase and DNA topoisomerase gene fragments show that although the cetacean poxviruses and the members of the orthopoxvirus genus originate from a common node, there is a clear divergence of the cetacean poxviruses into a unique branch. It is clear from both the bootstrapped and divergence phylograms, that there is a greater degree of divergence between the members of the Capripox genus, namely goatpox, sheeppox and lumpy skin disease viruses, than is observed between the two cetacean poxviruses. We infer that the cetacean poxviruses, as evidenced by their genetic isolation from all of the known

poxvirus genera, as well as the formation of a unique branch in the phylogenetic trees, should constitute a new genus within the Chordopoxvirinae subfamily of viruses.

The nucleotide and/or amino acid identity requirements for the classification of poxviruses as strains, species and genera vary depending on several factors. These factors include the gene from which the DNA sequence data was derived, the poxvirus genus under consideration and the length of the available DNA sequence. Becher et al. (2002), suggested the inclusion of sealpox virus as a new species of parapoxvirus based on nucleotide identities that ranged from 75 -79% when a 594-bp fragment of the major envelope protein was compared with homologous fragments from other ungulate parapoxviruses. Damaso et al. (2000), concluded that an emergent poxvirus, Cantagalo virus, constituted a strain of vaccinia virus and not a separate species of orthopoxvirus based on 98% nucleotide identity of a 950-bp fragment of the hemagglutinin gene when compared to other vaccinia viruses. DNA sequences derived from highly conserved genes or regions within a gene, may have different requirements for the classification of strain, species and genus than genes or gene regions possessing lesser degrees of conservation. For the purposes of this study, we consider DNA polymerase and DNA topoisomerase gene sequences that possess a nucleotide and amino acid difference >10% when compared to homologous sequences of terrestrial poxviruses, as indication of a new genus within Chordopoxvirinae. Pairwise comparisons showing 90-100% nucleotide and amino acid identity between poxviruses within a genus are considered separate strains. Our pairwise comparisons suggest the existence of more than one strain of parapoxvirus occurring in Steller sea lions.

Whenever possible, skin lesions that yielded a positive PCR result were inoculated onto cell culture to attempt virus isolation. Numerous attempts have been made in our laboratories and by others (VanBressem et al., 1999), however, to date, there are no reports of a cetacean or Steller sea lion poxvirus being successfully isolated in cell culture. While parapoxviruses have been isolated from pinnipeds in primary cell culture of pinniped tissues (Osterhaus et al., 1990; Osterhaus et al., 1994; Nettlleton et al., 1995), attempts to isolate the virus from the positive samples diagnosed in our laboratories were unsuccessful. We attribute these difficulties to an apparent specificity of the cetacean poxviruses to grow only in cetacean skin cells, and suspect that the cell lines that were available in this study, did not adequately support viral growth. Another possible cause for the difficulty in growing these cetacean poxviruses include the low amounts of viable poxvirus that may have been contained in the few available skin lesion samples. The parapoxvirus isolated by Nettleton et al. (1995), was grown in primary grey seal kidney cells, and was passed weekly over 25 days. Osterhaus et al. (1990) reported the use of primary harbor seal kidney cells to isolate an orthopox and a parapoxvirus from grey seal skin lesions. However, the orthopoxvirus "was lost" after several passages in culture. While poxviruses have been thoroughly reported in cetaceans and pinnipeds and repeated attempts to isolate those viruses have been made, relatively few successes, if any, are reported. Possible explanations for the difficulties in isolating this virus are poor sample quality, use of improper cell lines, and lack of ideal media and tissue culturing conditions, in general.

Only in recent years, has there been an advancement in the understanding of the genetic characteristics and evolutionary relationships of poxviruses, enabled by the

sequencing of complete poxvirus genomes and improvements in phylogenetic analysis. The genetic properties and phylogenetic relationships of poxviruses that affect marine mammals are still relatively unknown, as these viruses are difficult to isolate and typically, are found in samples that are not readily accessible. Further efforts to isolate poxviruses from cetacean and pinniped skin lesions are necessary for the advancement in the characterization of these viruses. The isolation of marine mammal poxviruses would permit the complete sequencing of the viral genome, development of new assays such as ELISA, and the simple detection of antibody responses in infected animals. It would also be possible to target full genes and develop a more detailed understanding of the structure and function of the proteins they encode. The PCR assays developed as part of this study will help to rapidly identify cetacean and pinniped pox and parapox viruses that afflict cetaceans and pinnipeds. The DNA sequences generated from poxvirus and parapoxvirus after the various PCR assays reported here, constitute a significant advancement in the molecular genetics of marine poxviruses and represent the first known report of comprehensive sets of nucleotide and amino acid sequences of novel poxviruses of cetacean and pinniped pox- and parapoxviruses.

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BIOGRAPHICAL SKETCH

Alexa Justine Bracht, the only child of Camille and Susan Bracht, was born in Boston, MA, 1977. Her family returned to New York in 1978, where Alexa spent the next 17 years of her youth. She began her college career at the University of Massachusetts- Amherst, where she majored in animal science. Exposed to many new animal husbandry experiences at UMASS, including vaccinating a herd of cattle at Greensborough county jail, delivering twin lambs (Lucy and Ethel), and castrating quite a few squealing piglets, Alexa decided her future would hold a life dedicated to animal service. Upon graduation with a Bachelor of Science, she relocated to Brigantine, NJ, for a job as a marine mammal stranding technician at the Marine Mammal Stranding Center. After countless seal rehabilitations and many cetacean strandings, she and her significant other Ethan, decided it was time to move on to bigger and sunnier places, and found them selves in Palm Coast, FL. After a year of working at a fine veterinary hospital, Alexa embarked on the pursuit of higher education as a graduate student at the University of Florida. Thrilled to find a professor involved in Florida's marine mammal stranding network, she joined the virology lab of Dr. Carlos Romero and began a study of poxvirus infection in cetaceans and pinnipeds. Over two and a half years, she gained a wealth of knowledge in virology, learned valuable molecular techniques, and made fabulous lifelong friends. After graduation, she plans to take a year off from her weekly, and sometimes daily, commute from Gainesville to Palm Coast, and return home to her husband, where they will await the arrival of their new baby together.