Discovery and characterization of novel papillomaviruses in Weddell seals around the Ross sea

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Abstract

Antarctica has become a rich area of research over the last few decades, and there has been a fair amount of research that has focused on the wildlife that inhabit this extreme environment. Within this ecosystem seals are high trophic level predators that provide important insight to the health of the environment. While our knowledge of the viruses circulating among Antarctic animals is limited, this is especially the case for Antarctic pinnipeds. This thesis highlights that our understanding of Antarctic animal virology is changing, albeit slowly. The application of metagenomics and development of high throughput sequencing has allowed for the discovery of novel viruses in this area, particularly around the Ross Sea and McMurdo Sound where research efforts have been concentrated.

The aim of this research was to assess the diversity of papillomaviruses in vaginal swabs collected from 81 female Weddell Seals over the summer field seasons of 2014-2016. Using metagenomic approaches seven papillomavirus genomes were identified andrecovered from this sample set. These viruses were highly diverse with six representing novel species and distinct evolutionary lineages within the family *Papillomaviridae.* This discovery extends our knowledge of viruses circulating among Antarctic animals that inhabit McMurdo Sound and the Ross Sea, which may offer support for monitoring the health of this ecosystem especially under conditions of environmental change.

Introduction

Marine mammals are regarded as sentinel species for monitoring ecosystem health because these are high trophic level predators with long life spans and large fat stores that act as depos for anthropogenic toxins (Kucklick et al., 2002; Mos et al., 2006). Looking at microbial diversity among these animals can reveal exposure to potential pathogens in the environment (Bossart, 2011; Mos et al., 2006). Furthermore, changes in response to certain infectious organisms / entities may be indicative of immunosuppression. This impact may be due to environmental stress and therefore is important for monitoring environmental changes (Acevedo-Whitehouse and Duffus, 2009). Viral pathogens have caused or been associated with several mortality events and disease in pinniped populations around the globe. From outbreaks of influenza virus in populations of harbour seals (*Phoca vitulina*) (Anthony et al., 2012; Bodewes et al., 2015; Mamaev et al., 1996; Zohari et al., 2014) to herpesvirus infections associated with high mortality in young harbour seals and cancer in California sea lions (*Zalophus californianus*) (King et al., 2002; King et al., 2001; Osterhaus et al., 1985). Morbilliviruses have caused several mass mortality events among pinniped populations. Namely, canine distemper virus (CDV) outbreaks in Baikal seals (*Phoca sibirica*) (Butina et al., 2010; Mamaev et al., 1996), Caspian seals (*Phoca caspica*) (Kennedy et al., 2000), harbour seals (Jensen et al., 2002; Osterhaus et al., 1990) and even crabeater seals in Antarctica (Laws and Taylor, 1957). Phocine distemper virus (PDV) has also been responsible for mortality among harbour seals (Jensen et al., 2002). While morbillivirus was detected in grey (*Halichoerus grypus*), harp (*Pagophilus groenlandica*), hooded (*Cystophora* cristata) and ringed (*Phoca hispida*) seals it has not been associated with as severe a mortality rate as in other pinnipeds (Duignan et al., 1997; Duignan et al., 1995). It has been suggested that under some circumstances, susceptibility to viral pathogens is exacerbated by exposure to environmental toxins (Ross et al., 2003).

The increase in metagenomics sequence data over the last decade has allowed for the identification of viruses whose role and pathogenicity is not yet understood. Adenoviruses have been associated with hepatitis of stranded California sea lions (Goldstein et al., 2011; Wright et al., 2015) and mortality of a captive California sea lion, South American fur seal (*Arctocephalus australis*), and South African fur seal

(*Arctocephalus pusillus*) all kept at the same zoo (Inoshima et al., 2013). Adenoviruses have also been identified in a zoo captive Hawaiian monk seal (*Neomonachus schauinslandi*) (Cortes-Hinojosa et al., 2016) and free-range stranded Northern elephant seals (*Mirounga angustirostris*), pacific harbour seals (*Phoca vitulina richardsii*) (Wright et al., 2015), South American fur seals and Sub Antarctic fur seals (*Arctocephalus tropicalis*) (Chiappetta et al., 2017). Parvoviruses have been identified in organ and brain samples of harbour seals, South American fur seals and sub Antarctic fur seals (Bodewes et al., 2013; Kluge et al., 2016). Small circular DNA viruses have been identified in faeces of a New Zealand fur seal (*Arctocephalus forsteri*) (Sikorski et al., 2013) and putative circoviruses in South American and sub Antarctic fur seals (Chiappetta et al., 2017).

To date, Antarctic seal associated viruses have been extremely understudied with the majority of viruses identified based on serological approaches. Within the last year, however, a polyomavirus (WsPyV) was identified in the kidney tissue of a deceased Weddell seal (*Leptonychotes Weddellii*) on the sea ice around McMurdo Sound (Varsani et al., 2017). Polyomaviruses have been detected in other pinnipeds including a California sea lion with T-cell lymphoma, a captive Hawaiian monk seal and placenta of a northern fur seal (*Callorhinus ursinus*) pup (Colegrove et al., 2010; Cortes-Hinojosa et al., 2016; Duncan et al., 2013; Wellehan et al., 2011). Anelloviruses are widespread and persistent among seals, identified in sub Antarctic fur seals, South American fur seals, harbour seals and California sea lions (Bodewes et al., 2013; Fahsbender et al., 2015; Kluge et al., 2016; Ng et al., 2011). Anelloviruses (TTLwV1 & 2) have also recently been recovered from Weddell seals around the McMurdo Sound and Ross Sea region (Fahsbender et al., 2017).

While several pinniped species inhabit sub-Antarctic islands and surrounding areas, only four species live on Antarctica and breed on the associated pack and fast ice. These include Weddell, leopard (*Hydrurga leptonyx*), crabeater (*Lobodon carcinophagus*) and Ross seals (*Ommatophoca rossii*). Weddell seals are the most southerly distributed seal species with a population of about 730,000- 800,000 (Erickson and Hanson, 1990) and they inhabit three regions of Antarctica: McMurdo Sound, Vestfolds and Signy Island (Testa et al., 1990). Weddell seals around McMurdo Sound have been studied the most extensively since the 1960s (Kooyman, 1965; Stirling, 1969). The sedentary, passive nature of Weddell seals combined with their proximity to research bases in McMurdo Sound (McMurdo Station, USA and Scott Base, New Zealand) has

allowed detailed life histories and studies of their physiology that continue today (Hückstädt et al., 2017; Siniff et al., 1977; Stirling, 1969). Weddell seals are unique from other Antarctic seals in that they breed on Antarctic fast ice, that is ice "fastened" to and extending out from the shoreline, where they remain over winter, making holes in the ice. These breathing holes also allow Weddell seals to forage for a wide range of prey under the ice such as different species of fish including Antarctic silverfish (*Pleuragramma antarcticum*), Bald notothen (*Pagothenia borchgrevinki*) and *Trematomus* spp, and cephalopods such as squid (Ainley and Siniff, 2009; Burns et al., 1998; Burns, 1999; Goetz et al., 2017; Green and Burton, 1987; Stirling, 1969).

Unlike leopard and crabeater seals, Weddell seals have a high tendency to return to breeding sites year after year and there is very little evidence of migration between colonies (Davis et al., 2008). This has allowed individuals to be tracked over different field seasons, as they can be expected to return to the same place each year. Breeding season of Weddell seals is between October and December where females aggregate together in breeding colonies, likely increasing the mating success of males (Stirling, 1969). Pupping takes place in congregated colonies of females hauled out on the fast ice around October to give birth, pups are weaned for about 6 weeks and quickly learn to swim (Burns et al., 1999; Stirling, 1969; Testa and Siniff, 1987). Despite the extremely harsh Antarctic winter, Weddell seals have adapted to surviving in these conditions (Heerah et al., 2017).

During early Antarctic expeditions, Weddell seals around Ross Sea were harvested to feed dog teams brought with expedition groups. However, in 1996 dogs were banned from Antarctica after a mass mortality of crabeater seals suspected to have been infected with canine distemper virus that could be transmitted from dogs (Laws and Taylor, 1957). This raised concerns to the anthropogenic impact on the ecosystem and led to research investigating potential pathogens and toxins introduced to the environment (Kerry and Riddle, 2009). Since then the increasing awareness of conservation in Antarctica has led to the Commission for the Conversation of Antarctic Marine Living Resources (CCAMLR) approving the Ross Sea as the world's largest marine protected area. Of the 730,000-800,000 Weddell seals in Antarctica, about 32,000- 50,000 are estimated to inhabit the Ross Sea (Ainley, 1985). As a high trophic level predator occupying this area year around Weddell seals play a vital role in this ecosystem. It

follows that much of the research in the Ross Sea has continued to monitor the behaviour and physiology of this population. These extensive studies of a sentinel species provides important insight to impacts of environmental change on the marine environment (Hückstädt et al., 2017).

In the last decade, with the introduction of metagenomics approaches and high throughput sequencing our understanding of microbes in this area has dramatically increased and led to the classification of several novel viruses. The Varsani research group in collaboration with that of Jennifer Burns have been the main contributors to understanding pinniped-associated associated viruses in the Ross Sea and McMurdo Sound. Until recently, the majority of these studies have looked at avian species and only in the past year have two novel viruses been classified in Weddell seals inhabiting the Ross sea (Fahsbender et al., 2017; Varsani et al., 2017).

Over the last three Austral summer field seasons (2014-2016), vaginal swab samples from Weddell seals on the fast ice on in the McMurdo sound have been collected by Jennifer Burns and her research group specifically for viral research. The research team from the University of Anchorage, USA included Michelle Shero, Roxanne Beltran, Amy Kirkham and Greg Frankfurter and the samples were taken under the National Marine Fisheries Service Marine Mammal permit #17411, Antarctic Conservation Act permit #2014-003, and University of Alaska Anchorage's Institutional Animal Care and Use Committee approval #419971. Preliminary results from next generation sequencing of 2014/2015 collected samples identified papillomavirus-like sequences in vaginal swabs. This prompted investigation the recovery of these viruses as well as further sampling of individuals in the following seasons.

Papillomaviruses are a highly diverse family of dsDNA viruses found in a very wide range of hosts, however, they have only been characterised in one other pinniped species, a California sea lion (Rivera et al., 2012). Papillomaviruses have co-evolved with their host with some host switching, adaptive radiation, recombination and positive selection also contributing to their divergence (Bravo and Alonso, 2007; Burk et al., 2013; García-Vallvé et al., 2005; Gottschling et al., 2007; Varsani et al., 2006). In Antarctica, only two papillomaviruses have been characterised in the last five years are those among Adelie penguins (Van Doorslaer et al., 2017; Varsani et al., 2014). As part of this MSc thesis research we aimed to identify and characterise novel papillomaviruses in Weddell

seals inhabiting the Ross Sea and McMurdo Sound. Further we will comparatively analyse these with known papillomaviruses from seal and other host species. This MSc research expands our knowledge of papillomaviruses diversity among Antarctic pinnipeds.

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Viruses associated with Antarctic wildlife: from serology based detection to identification of genomes using high throughput sequencing

Abstract

The Antarctic, sub-Antarctic islands and surrounding sea-ice provide a unique environment for the existence of organisms. Nonetheless, birds and seals of a variety of species inhabit them, particularly during their breeding seasons. Early research on Antarctic wildlife health, using serology-based assays, showed exposure to viruses in the families *Birnaviridae*, *Flaviviridae*, *Herpesviridae*, *Orthomyxoviridae* and *Paramyxoviridae* circulating in seals (Phocidae), penguins (Spheniscidae), petrels (Procellariidae) and skuas (Stercorariidae). It is only during the last decade or so that polymerase chain reaction-based assays have been used to characterize viruses associated with Antarctic animals. Furthermore, it is only during the last five years that full/whole genomes of viruses (adenoviruses, anelloviruses, orthomyxoviruses, a papillomavirus, paramyoviruses, polyomaviruses and a togavirus) have been sequenced using Sanger sequencing or high throughput sequencing (HTS) approaches. This review summaries the knowledge of animal Antarctic virology and discusses potential future directions with the advent of HTS in virus discovery and ecology.

1. Introduction

Among Earth's oceans, those in the Polar Regions are the smallest and most constrained, the Arctic Ocean encircled by landmasses and the Southern Ocean by the Antarctic Circumpolar Current (ACC). The latter ocean is bounded to its north by the Antarctic Polar Front (APF), a well-known faunal barrier, and has a high degree of endemism among both vertebrates and invertebrates (e.g. (Briggs, 1995; Eastman, 2013). Owing to dramatic annual cycles of heat and light, the productivity of the Southern Ocean is highly constrained on a seasonal basis, a characteristic that provides a generally challenging environment for the existence of organisms. Moreover, the high latitude Southern Ocean, i.e. that portion south of the Southern Boundary of the ACC (SBACC), is covered by sea ice for much of the year, in some places the entire year. Most of the birds and seals of a variety of species that inhabit that zone are endemic and resident, the most unvarying species assemblage found in Southern Hemisphere oceans; only a few migrant species augment that assemblage during summer (Ribic and Ainley, 1989). The species comprised this assemblage breed either on Antarctic islands (birds) or in the sea ice that surrounds the continent (seals). In contrast, waters north of the SBACC host a much more speciose, seasonally varying seabird and marine mammal assemblage composed of species breeding on low latitude islands bordering the APF (Antarctic and sub-Antarctic) as well as seasonal migrants from more temperate regions (e.g. (Ainley et al., 1994; Laws, 1977a; Laws, 1977b; Ribic and Ainley, 1989; Ribic et al., 2011). In accord with trends of diversity varying inversely with latitude, overall diversity of vertebrate species is low in the Southern Ocean, especially south of the SBACC, but populations are immense (e.g. (Laws, 1977a; Laws, 1977b).

Inhabiting the pack-ice surrounding Antarctica is a unique assemblage of pagophilic seals, crabeater (*Lobodon carcinophaga*), leopard (*Hydrurda leptonyx*), Ross (*Ommatophoca rossii*) and Weddell seal (*Leptonychotes weddellii*). Weddell seals colonize near-shore fast-ice regions feeding mainly on fish, while other Antarctic seals remain year round in the pack-ice composed of individual, often compacted floes. Crabeater seals feed principally on krill (*Euphausia* spp.), and Ross seals mostly on squid.

Unlike the other Antarctic seals, leopard seals are solitary and highly predatory feeding on penguins and other seals as well as fish, krill and cephalopods (Siniff, 1981). Southern elephant seals (*Mirounga leonina*) occupy sub-Antarctic islands for breeding, then migrate south to Antarctica, some hauling out on land for moulting; Antarctic fur seals (*Arctocephalus gazella*) breed on peri-Antarctic islands, such as Macquarie, South Georgia and South Sandwich, as well as islands of the northern Antarctic Peninsula, around which they also hunt for krill and fish (Siniff et al., 2008).

Confined to pack-ice affected waters south of the SBACC are the Adélie (*Pygoscelis adeliae*) and emperor (*Aptenodytes forsteri*) penguins; north of that boundary are three other penguin species: gentoo (*P. papua*), chinstrap (*P. antarctica*) and macaroni (*Eudyptes chrysolophus*) (Williams, 1995). While mostly distributed to the north, populations of chinstrap, gentoo and macaroni penguins breed on islands of the northwestern Antarctic Peninsula, overlapping with Adélie penguins. Gentoo penguins are distributed as far as temperate waters surrounding the Falkland Islands, while macaroni penguins mainly colonize Heard and South Georgia islands in proximity to the APF (Trathan et al., 2016). Colonies of king penguins (*A. patagonicus*), royal penguins (*E. schlegeli*) and rockhopper penguins (*E. chrysocome*) are found on sub-Antarctic islands and do not inhabit the coastal Antarctic continent. Flying birds inhabiting Antarctica and sub-Antarctic regions include skuas, petrels, terns, gulls and Albatross (Brooke, 2004; Murphy, 1936).

In addition to the two penguins endemic to the sea ice zone are the endemic snow (*Pagodroma nivea*) and Antarctic petrels (*Thalassoica antarctica*), this pagophilic community increased only in summer by a few other flighted seabirds: skuas, albatross and a few petrel species. The peri-Antarctic and sub-Antarctic islands, and surrounding ice-free ocean, are densely populated by many more petrel and albatross species, with sparse inclusion of skuas and larid species (Brooke, 2004; Murphy, 1936).

2. *Early reports of mass mortality in Antarctic animals*

Due to its zoogeographical isolation, introduction of pathogens and parasites to these populations of Antarctic wildlife, particularly the endemics of high latitude, may have detrimental effects. Understanding these potential effects required a knowledge of the entities circulating in the ecosystem. This concern drove interest toward detecting viruses, bacteria and parasites among Antarctic animals providing insight to their health. There have been a few reported cases of mass mortality where the disease-causing agent was undetermined. In 1971, several hundred gentoo penguin chicks at a Signey Island colony, South Orkneys, were found dead (MacDonald and Conroy, 1971). Although symptoms were described as similar to puffinosis coronavirus infection, no isolation of the disease agent was possible (Barbosa and Palacios, 2009). In 1972, about 65% of Adélie penguin chicks were reported dead in a colony near Mawson Station. In this case, penguins were found face down and apparently unable to walk or stand properly (Kerry et al., 2009). The cause of this disease was not determined. The only mass mortality reported in seals was the death of over 1500 crabeater seals in a colony around Crown Prince Gustav Channel, Antarctic Peninsula in 1955 (Laws and Taylor, 1957). Interestingly, Weddell seals in the area were unaffected and while the nature of this disease was unknown, a viral infection was suggested. Laws and Taylor (1957) noted that the population of seals in this area was almost ten times higher than normal and predicted that this crowding and partial starvation likely contributed to the effects of the disease.

3. *Viruses associated with Antarctic animals*

Only within the last ten years has an increase in knowledge of the viral diversity been evident among Antarctic wildlife at a genome level. Early work, beginning in the mid-1970s, on identifying viruses associated with Antarctic animals relied on serology-based assays (Table 1). The research then was particularly focused around detecting pathogens posing a risk to animal health due to concerns regarding the impact of increased anthropogenic activity, e.g. research, tourism, on birds and marine mammals (Kerry and

Riddle, 2009). During the early period of Antarctic virus research, paramyxoviruses, orthomyxoviruses, birnaviruses, herpesviruses and flaviviruses were serologically detected (Table 1, Figure 1). Subsequently, between 2000 and 2010, probe based assays using polymerase chain reaction (PCR) were used to detect paramyxoviruses, orthomyxoviruses and a poxvirus (Table 2, Figure 1). Finally, in the last decade, advancements in high throughput sequencing (HTS) approaches are beginning to have an impact on our knowledge of Antarctic animal virology. For example, in the last five years using viral metagenomic based approaches with HTS, various novel viruses have been identified and characterized at a genomic level (Table 2, Figure 1). These include adenoviruses, anelloviruses, orthomyxoviruses, papillomaviruses, polyomaviruses and paramyxoviruses.

Table 1: Summary of Antarctic bird- and mammal-associated viruses detected through serological approaches.

ID	Virus taxonomy				Notes					
	Order	family	genus	Virus	Host	Method	Sample	Location	Year of collection	Reference
1	*Mononegavirales*	*Paramyxoviridae*	*Avulavirus*	Avian paramyxovirus	Adelie penguins (*Pygoscelis adeliae*)	Hemagglutination-inhibition, immunodiffusion tests, morphology	2/42 serum samples with antibodies to NDV, cloacal swabs	Wilkes base	-	(Morgan and Westbury, 1981)
2						Hemagglutination-inhibition, immunodiffusion tests, morphology	serum, 2 APMV viruses isolated from 550 cloacal swabs	Peterson Island, Midgley Island, Shirley Island, Cameron Island, d'Urville (casey station)	-	(Morgan and Westbury, 1981)
3						Virus isolation, Haemagglutination test	Serum samples and cloacal swabs	Vestfold Hills	December 1981	(Morgan and Westbury, 1988)
4						Indirect ELISA, electron microscopy	Cloacal swab, serum	Ross Island	1978	(Austin and Webster, 1993)
5					Royal penguins (*Eudyptes chrysolophus*)	Hemagglutination-inhibition, immunodiffusion tests, morphology	Cloacal samples	Macquarie Island	-	(Morgan et al., 1981)
5					King penguin (*Aptenodytes patagonicus*)	Hemagglutination-inhibition, immunodiffusion tests, morphology	Cloaca samples	Macquarie Island	-	(Morgan et al., 1981)
7					South Polar skua (*Stercorarius maccormicki)*	Indirect ELISA, electron microscopy	Serum	Ross Island	1978, 1986	(Austin and Webster, 1993)
8						Haemagglutination-inhibition test	Serum samples and cloacal swabs	Davis station	November / December 1999	(Miller et al., 2008)
9			*Morbillivirus*	Canine distemper virus	Leopard seal (*Hydrurda leptonyx*)	CDV-like antibodies detected through microneutralization test using two CDV strains and a phocine distemper virus isolate	2/3 serum samples tested positive	Antarctic peninsula	1989	(Bengtson et al., 1991)
10					Crabeater seal (*Lobodon carcinophaga*)	CDV-like antibodies detected through microneutralization test using two CDV strains and a phocine distemper virus isolate	35% serum samples tested positive for CDV	Antarctic peninsula	January / March 1989	(Bengtson et al., 1991)
11	Unassigned	*Orthomyxoviridae*	*Influenzavirus*	Influenza A virus	Adelie penguins (*Pygoscelis adeliae*)	Hemagglutination-inhibition, immunodiffusion tests	Serum	Peterson Island (Casey)	-	(Morgan et al., 1981)
12						Hemagglutination-inhibition, Neuraminidase-inhibition tests	Serum	Ross Island	1978	(Austin and Webster, 1993)
13						Hemagglutination-inhibition test against H1N1, H3N2, H5N1, and H7N2 antigens	Serum	Hope Bay	December-March, 1998, 2001, and 2002	(Baumeister et al., 2004)

14				South Polar skua (*Stercorarius maccormicki)*	Indirect ELISA, Hemagglutination-inhibition tests	Serum	Ross Island	1978, 1986	(Austin and Webster, 1993)
15					Capture ELISA	Serum and cloacal swabs	Davis station	November / December 1999	(Miller et al., 2008)
16					Hemagglutination inhibition test against H1N1, H3N2, H5N1, and H7N2 antigens	Serum	Potter peninsula and Hope Bay	December-March, 1998, 2001, and 2002	(Baumeister et al., 2004)
17				Chinstrap penguins (*Pygoscelis antarctica*)	Hemagglutination inhibition test against H1N1, H3N2, H5N1, and H7N2 antigens	Serum	Potter peninsula	December-March, 1998, 2001, and 2002	(Baumeister et al., 2004)
18				Gentoo penguins (*Pygoscelis papua*)	Hemagglutination inhibition test against H1N1, H3N2, H5N1, and H7N2 antigens	Serum	Potter peninsula	December-March, 1998, 2001, and 2002	(Baumeister et al., 2004)
19					Influenzavirus type A virus antibody ELISA kit		Bird Island	1996	
20				Giant petrel (*Macronectes giganteus*)	Hemagglutination inhibition test against H1N1, H3N2, H5N1, and H7N2 antigens	Serum	Potter peninsula and Harmony peninsula	December-March, 1998, 2001, and 2002	(Baumeister et al., 2004)
21	*Birnaviridae*	*Avibirnavirus*	Infectious bursal disease virus	Adelie penguins (*Pygoscelis adeliae*)	Virus neutralization tests, IBDV serotype 1 and 2 antibodies	High titer of neutralizing antibodies detected in 2.6% or 133 penguins	Mawson station	1995/96 summer	(Gardner et al., 1997)
22					Virus neutralization tests to measure antibody titers to IBDV serotype 1	Seroprevalence 7.7%, no significant difference between locations or years. Highly significant titres were obtained from 1.8% of birds	Mawson coast, Davis Coast, Terra Nova Bay	November-February 1996-2002	(Watts et al., 2009)
23				Emperor penguin (*Aptenodytes forsteri*)	Virus neutralization tests, IBDV serotype 1 and 2 antibodies	High titer of neutralizing antibodies detected in 65.4% of 53 penguins	Mawson station	1995/96 summer	(Gardner et al., 1997)
24					Virus neutralization tests to measure antibody titers to IBDV serotype 1		Auster Rookery, Amanda Bay rookery, Cape Washington rookery	November - February 1996-2001	(Watts et al., 2009)
25				King penguin (*Aptenodytes patagonicus*)	Virus neutralization tests, IBDV serotype 1 and 2 antibodies, cough and conjunctivitis clinical signs	Serum of adults and chicks	sub-Antarctic Iles Crozet	November 1996-February 1997	(Gauthier-Clerc et al., 2002)
26				South Polar skua (*Stercorarius maccormicki)*	Antibody neutralization test	Serum and cloacal swab	Davis station	November/December 1999	(Miller et al., 2008)

27						Virus neutralization tests to measure antibody titers to IBDV serotype 1	Antibodies detected in 11.8% of individuals, Significant difference in prevalence between sample years	Vestfolds, Davis station	November-February 1999-2002	(Watts et al., 2009)
28		*Flaviviridae*	*Flavivirus*	-	South Polar skua (*Stercorarius maccormicki)*	Capture ELISA	Serum samples and cloacal swabs	Davis station	November / December 1999	(Miller et al., 2008)
29	*Herpesvirales*	*Herpesviridae*	*Varicellovirus*	Phocid alphaherpesvirus 1	Ross seal (*Ommatophoca rossii*)	Indirect ELISA using PhHV-1 as antigen, serum neutralization test	Serum	Queen Maud Land	2001	(Tryland et al., 2012)
30					Crabeater seal (*Lobodon carcinophaga*)	Indirect ELISA using PhHV-1 as antigen, serum neutralization test	Serum	Queen Maud Land	2001	(Tryland et al., 2012)
31						Testing for neutralizing antibodies against phocine, feline and canine herspevirus using either microneutralization or by neutralizing peroxidase-linked antibody assay	Serum	Weddell Sea	1990	(Harder et al., 1991)
32					Weddell seal (*Leptonychotes weddellii*)	Indirect ELISA using PhHV-1 as antigen, serum neutralization test (SNT)	Serum	Queen Maud Land	2001	(Tryland et al., 2012)
33						Testing for neutralizing antibodies against phocine, feline and canine herspevirus using either microneutralization or by neutralizing peroxidase-linked antibody assay	Serum	Weddell Sea	1990	(Harder et al., 1991; Stenvers et al., 1992)
34					Antarctic fur seal (*Arctocephalus gazelle*)	Indirect ELISA using PhHV-1 as antigen	Serum	Bouvet Island	2000-2001,2001-2002	(Tryland et al., 2012)

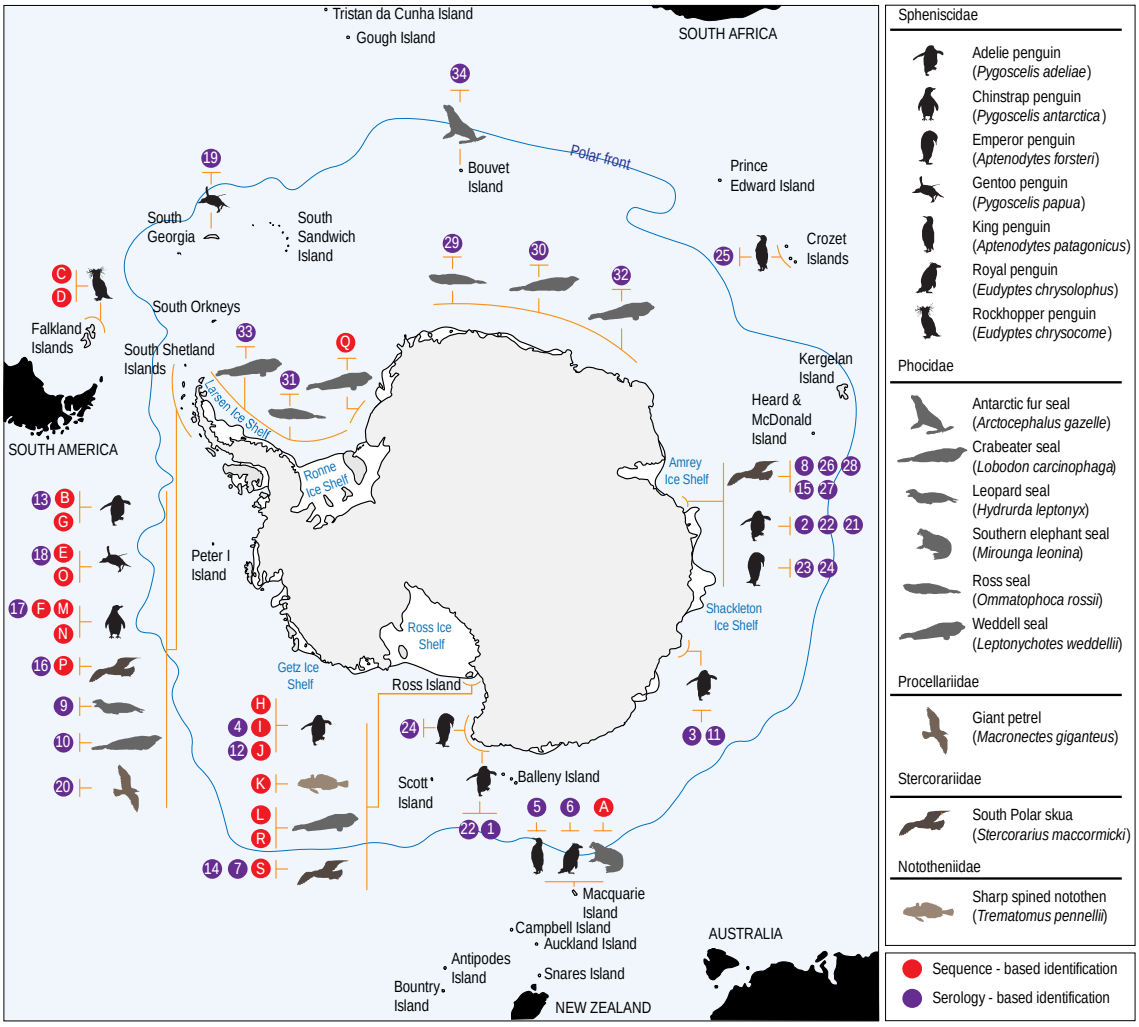
CDV: Canine distemper virus
ELISA: Enzyme-linked immunosorbent assay
IBDV: Infectious bursal disease virus

Table 2: Summary of Antarctic bird- and mammal-associated viruses identified through sequencing based approaches, including respective accession numbers of the partial and full genome sequences.

ID	Virus taxonomy			Host	Notes					
	Family	Genus	Species & virus		Method	Sample type	Location	Year of collection	Accession #	Reference
A	*Togaviridae*	*Alphavirus*	*Southern elephant seal virus* [Southern elephant seal virus (SES virus)]	Southern elephant seals (*Mirounga leonina*)	Virus cultured in BHK-21 cells virus from blood sucking lice *Lepidophthirus macrorhini* and used in viral neutralization assay for serology. Negative stain electron microscopy. RT-PCR and Sanger sequencing of capsid protein gene.	*Lepidophthirus macrorhini* and serum from southern elephant seal virus	Macquarie Island	-	AF315122 HM147990	(Forrester et al., 2012; La Linn et al., 2001)
B	*Paramyxoviridae*	*Avulavirus*	*Avian avulavirus 1* [New castle disease virus (NDV)]	Adélie penguins (*Pygoscelis adeliae*)	RT-PCR and real-time PCR targeting F gene of NDV, virus culture, haemagglutination test using antigen against B1 NDV strain, Sanger sequencing.	Cloacal/tracheal swabs and serum samples	King George Island	2006	HM143848 – HM143850	(Thomazelli et al., 2010)
C			*Avian avulavirus 10* [Avian paramyxovirus 10 (APMV10)]	Rockhopper penguins (*Eudyptes chrysocome*)	Real-time RT-PCR, hemagglutination assay, binaxNOW influenza A&B test, hemagglutination inhibition assay, ELISA, electron microscopy, Sanger sequencing.	Cloacal/tracheal swabs and serum samples	Falkland Islands	2007	HM147142, HM755886 – HM755888 (updated following the publication of Goraichuk et al. 2017)	(Miller et al., 2010)
D				Rockhopper penguins (*Eudyptes chrysocome*)	Complete genome sequencing using Illumina and Sanger sequencing.	Cloacal/tracheal swabs and serum samples	Falkland Islands	2007	HM147142, HM755886 - HM755888	(Goraichuk et al., 2017)
E			Unclassified [Avian paramyxovirus 15, 16, 17 (APMV15, APMV16, APMV17)]	Gentoo penguins (*Pygoscelis papua*)	Hemagglutination assay, RT-PCR and Sanger sequencing.	Virus isolated from 12 cloacal swabs, 5 confirmed by sequencing	Kopaitic Island	2014 - 2016	KY452442 - KY452444	(Neira et al., 2017)
F	*Orthomyxoviridae*	*Influenzavirus A*	*Influenzavirus A* [Avian Influenza A virus H5N5]	Chinstrap penguins (*Pygoscelis antarctica*)	RT-PCR, HTS, ELISA using nucleoprotein, hemagglutination assay.	Cloacal/tracheal swabs and serum samples	Antarctic peninsula	2015	GISAID #s [EPI774530-EPI774536, EPI774538-EPI774539]; [EPI774527- EPI774529]	(Hurt et al., 2016)
G			*Influenzavirus A* [Avian influenza A virus H11N2]	Adélie penguins (*Pygoscelis adeliae*)	RT-PCR, virus culture, ELISA, whole genome Sanger sequencing.	Cloacal/tracheal swabs and serum samples	Rada Covadonga, Antarctic Peninsula and King George Island	2013	KJ729348 – KJ729379	(Hurt et al., 2014)
H	*Papillomaviridae*	*Treisepsilonpapillomavirus*	*Treisetapapillomavirus 1* [Pygoscelis adeliae papillomavirus 1]	Adélie penguins (*Pygoscelis adeliae*)	HTS-informed approach, genome recovered by abutting primers, cloned and Sanger sequenced.	Faeces	Cape Crozier, Ross Island	2012 / 2013	KJ173785	(Varsani et al., 2014)

I			Unclassified [Pygoscelis adeliae papillomavirus 2]	Adélie penguins (*Pygoscelis adeliae*)	HTS-informed approach, genome recovered by abutting primers, cloned and Sanger sequenced.	Cloacal swab	Cape Crozier, Ross Island	2014	MF168943	(Van Doorslaer et al., 2017)
J	*Polyomaviridae*	*Gammapolyomavirus*	*Pygoscelis adeliae polyomavirus 1* [Adelie penguin polyomavirus (AdPyV)]	Adélie penguins (*Pygoscelis adeliae*)	HTS-informed approach, genome recovered by abutting primers, cloned and Sanger sequenced.	Faeces	Cape Royds, Ross Island	2012 / 2013	KP033140	(Varsani et al., 2015)
K		*unassigned*	*Trematomus pennellii polyomavirus 1* [Sharp-spined notothenia polyomavirus (SspPyV)]	Sharp spined notothen (*Trematomus pennellii*)	HTS-informed approach, genome recovered by abutting primers, cloned and Sanger sequenced.	Stomach and liver samples	Ross Sea	2012 / 2013	KP768176	(Buck et al., 2016)
L		*Betapolyomavirus*	*Leptonychotes weddellii polyomavirus 1* [Weddell seal polyomavirus (WsPyV)]	Weddell seal (*Leptonychotes weddellii*)	HTS-informed approach, genome recovered by abutting primers, cloned and Sanger sequenced.	Kidney	Ross Sea	2014	KX533457	(Varsani et al., 2017)
M	*Adenoviridae*	*Siadenovirus*	*Penguin siadenovirus A* [Chinstrap penguin adenovirus (CSPAdV)]	Chinstrap penguins (*Pygoscelis antarctica*)	PCR of protein VI and capsid protein hexon genes.	Lung, liver, kidney, heart, intestine, trachea samples	King George Island	2009/2010	KC593379 - KC593386	(Lee et al., 2014)
N			*Penguin siadenovirus A* [Chinstrap penguin adenovirus (CSPAdV)]	Chinstrap penguins (*Pygoscelis antarctica*)	RACE PCR, Sanger sequencing of whole genome.	Lung, liver, kidney, heart, intestine, trachea samples	King George Island	2008-2013	KP144329- KP144330	(Lee et al., 2016)
O			*Penguin siadenovirus A* [Gentoo penguin adenvirus (GPAdV)]	Gentoo penguins (*Pygoscelis papua*)	RACE PCR, Sanger sequencing of whole genome.	lung, liver, kidney, heart, intestine, trachea, feces	King George Island	2008-2013	KP279746 - KP279747	(Lee et al., 2016)
P			*Skua siadenovirus A* [South polar skua adenovirus 1 (SPSAdV 1)]	South Polar skua (*Stercorarius maccormicki)*	Nested PCR, RACE PCR, Sanger sequencing of whole genome.	kidney	King George Island	2007-2009	HM585353 (full genome) JM585354-HM585358	(Park et al., 2012)
Q	*Poxviridae*	*Parapoxvirus*	Unassigned [Seal poxvirus]	Weddell seal (*Leptonychotes weddellii*)	Electron microscopy, PCR of B2L gene, sequencing of B2L gene.	Neck skin lesion from a single seal	Queen Maud Land	2001	AJ622900	(Tryland et al., 2005)
R	*Anelloviridae*	unassigned	Unassigned [Torque teno Leptonychotes weddelli virus 1,-2(TTLwV1 & TTLwV2)]	Weddell seal (*Leptonychotes weddellii*)	HTS-informed approach, genome recovered by abutting primers, cloned and Sanger sequenced.	Vaginal, nasal and faecal samples	Ross Sea	November-February 2014-2015	KY246479 – KY246627	(Fahsbender et al., 2017)
S			Unassigned [Torque teno Leptonychotes weddelli virus 1 (TTLwV1)	South Polar skua (*Stercorarius maccormicki)*	HTS-informed approach, genome recovered by abutting primers, cloned and Sanger sequenced.	Faecal sample	Ross Sea	November / December 2014	KY246476 - KY246478	(Fahsbender et al., 2017)

cDNA: complementary DNA
ELISA: Enzyme-linked immunosorbent assay
PCR: Polymerase chain reaction
HTS: High throughput sequencing
RACE: Rapid Amplification of cDNA ends
RT-PCR: Reverse transcription PCR



Method of identification*	Order	Family	Genus	Species
29 30 31 32 33 34	*Herpesvirales*	*Herpesviridae*	*Varicellovirus*	*Phocid alphaherpesvirus 1*
1 2 3 4 5 6 7 8 B	*Mononegavirales*	*Paramyxoviridae*	*Avulavirus*	*Avian avulavirus 1*
C D	*Mononegavirales*	*Paramyxoviridae*	*Avulavirus*	*Avian avulavirus 10*
E	*Mononegavirales*	*Paramyxoviridae*	*Avulavirus*	unassigned (avian avulavirus 15, -16, -17)
9 10	*Mononegavirales*	*Paramyxoviridae*	*Morbillivirus*	*Canine distemper virus*
A	Unassigend	*Togavirdae*	*Alphavirus*	*Southern elephant seal virus*
M N O	Unassigned	*Adenoviridae*	*Siadenovirus*	*Penguin siadenovirus A*
P	Unassigned	*Adenoviridae*	*Siadenovirus*	*Skua siadenovirus A*
R	Unassigned	*Anelloviridae*	unassigned	unassigned (torque teno Leptonychotes weddelli virus 1, -2)
S	Unassigned	*Anelloviridae*	unassigned	unassigned (torque teno Leptonychotes weddelli virus 1)
21 22 23 24 25 26 27	Unassigned	*Birnaviridae*	*Avibirnavirus*	*Infectious bursal disease virus*
28	Unassigned	*Flaviviridae*	*Flavivirus*	-
11 12 13 14 15 16 17 18 19 20 F G	Unassigned	*Orthomyxoviridae*	*Influenzavirus A*	*Influenza virus A*
H	Unassigned	*Papillomaviridae*	*Treisepsilonpapillomavirus*	*Treisetapapillomavirus 1*
I	Unassigned	*Papillomaviridae*	unassigned	unassigned (pygoscelis adeliae polyomavirus 2)
L	Unassigned	*Polyomaviridae*	*Betapolyomavirus*	*Leptonychotes weddellii polyomavirus 1*
J	Unassigned	*Polyomaviridae*	*Gammapolyomavirus*	*Pygoscelis adeliae polyomavirus 1*
K	Unassigned	*Polyomaviridae*	unassigned	*Trematomus pennellii polyomavirus 1*
Q	Unassigned	*Poxviridae*	*Parapoxvirus*	unassigned (seal poxvirus)

Figure 1: Distribution of viruses identified among populations of animals of the Antarctic and high latitude sub-Antarctic. Colored circles denote the method of viral identification. Purple indicates serology based identification, number inside circle corresponding to details provided in Table 1. Red circle indicates sequence based identification, letter inside circle corresponding to details provided in Table 2.

3.1 Positive sense RNA viruses

3.1.1 *Flaviviridae*

Flaviviridae is a family of enveloped positive sense RNA viruses with four genera: *Flavivirus*, *Hepacivirus*, *Pegivirus* and *Pestivirus*. Their genomes range in length from 8.9-13 kb (Simmonds et al., 2017b). While no genomic information is available for flaviviruses circulating in Antarctic animals, neutralizing antibodies to a flavivirus were detected in the serum of South Polar skuas (*Stercorarius maccormicki*) around Davis station, East Antarctica (Miller et al., 2008). As lower latitude seabirds are infested with ticks (e.g. (Lee and Baust, 1987), which are known to carry flaviviruses, this may indicate the transmission of tick-borne flaviviruses to seabirds, especially given the fact that a flavivirus was isolated from seabird ticks (*Ixodes uriae*) infecting king penguins on Macquarie Island (Major et al., 2009).

3.1.2 *Togaviridae*

The family *Togaviridae* consist of enveloped positive sense RNA viruses with a genome length of about 11-12 kb in length. Togaviruses are classified into two genera, *Alphavirus* and *Rubivirus* (Power et al., 2017). While the human pathogenic virus, rubella virus, is the only known member of the single species in the genus *Rubivirus* to date (species *Rubella virus*), all animal togaviruses are classified as alphaviruses (Power et al., 2017). The life cycle of alphaviruses requires an arthropod vector, either a mosquito or tick, for transmission to their vertebrate host. The first instance of alphaviruses found in marine mammals was shown in southern elephant seals of Macquarie Island. Initially the seal alphavirus was isolated from the blood-sucking louse (*Lepidophthirus macrorhini*) that is widespread among southern elephant seals. However, a high seroprevalence of antibodies against the southern elephant seal alphavirus strongly indicated its transmission from lice to seals (La Linn et al., 2001). The full genome of this alphavirus (Southern elephant seal virus) was determined in a later study (Forrester et al., 2012).

3.2 Negative sense RNA viruses

3.2.1 *Orthomyxoviridae*

Viruses in the family *Orthomyxoviridae* have enveloped, negative sense RNA genomes that consist of 6-8 segments. Seven genera are established in this family (*Influenzavirus A, Influenazavirus B, Influenzavirus C, Influenzavirus D, Isavirus, Quaranjavirus* and *Thogotovirus*) (McCauley et al., 2011) . *Influenza A virus* is the only species in the genus *Influenzavirus A*, consisting of several pathogenic strains infecting humans, horses, pigs, whales, seals, birds and mink(McCauley et al., 2011). Influenza A virus strains are transmissible to humans and have caused worldwide endemics. This zoonotic nature of influenza viruses has led to extensive research on Influenza A virus. Antarctica continues to provide an interesting environment to study Influenza A viruses, especially in the case of migratory birds. For example, the South Polar skuas that breed on the Antarctic continent in the summer but move north, well into the Northern Hemisphere during the non-breeding season (Weimerskirch et al., 2015), thus act as potential vectors bringing in new variants and reassortants to Antarctica during each breeding season.

The majority of studies screening for influenza have used hemagglutination-inhibition assays to detect antibodies against several common strains of avian influenza virus circulating among Antarctic animals. Antarctic influenza virus research carried out between 1978 and 2002, detected antibodies against several strains of avian influenza virus in South Polar skuas, southern giant petrels (*Macronectes giganteus*), and Adélie, chinstrap and gentoo penguins from various locations around the Antarctic Peninsula, Ross Island and East Antarctica (Table 1, Figure 1) (Austin and Webster, 1993; Baumeister et al., 2004; Miller et al., 2008; Morgan and Westbury, 1981). Such research has indicated that influenza virus is highly widespread and prevalent in Antarctic birds. However, the pathogenicity of avian influenza virus in these populations is unknown. No genomic information for Influenza A viruses in Antarctic birds was available until Hurt et al. (2014) used HTS approaches to identify influenza A virus (H11N2) in Adélie

penguins around the Antarctic Peninsula. A following study around the same area later identified the strain H5N5 among chinstrap penguins (Hurt et al., 2016).

None of the early studies have detected antibodies against influenza A virus strains in Antarctic pinnipeds, although several studies have looked at crabeater and Weddell seals (Austin and Webster, 1993; McFarlane, 2009).

3.2.2 *Paramyxoviridae*

Paramyxoviridae is a family of enveloped, non-segmented negative sense RNA viruses in the order *Mononegavirales* with genomes of ~15 kb. Paramyxoviruses are divided into seven genera: *Aquaparamyxovirus, Ferlavirus, Respirovirus, Morbillivirus, Rubulavirus, Henipavirus,* and *Avulavirus*. The genus *Avulavirus* includes of 13 formally classified species of avian paramyxoviruses including avian paramyxovirus 1 (AVPM-1) (Afonso et al., 2016). The genus *Morbillivirus* contains paramyxoviruses infecting mammals.

The majority of research on paramyxoviruses in Antarctica has been based on serological studies using haemagglutination inhibition assay to detect antibodies against paramyxoviruses in serum samples (Table 1). A high prevalence of antibodies to NDV in South Polar skuas has been reported (Miller et al., 2010), whereas low incidences have been found in Adélie and royal penguins around coastal Antarctica and Macquarie Island (Table 1, Figure 1) (Morgan and Westbury, 1981; Morgan and Westbury, 1988). So far, king, gentoo and rockhopper penguin colonies on Macquarie Island have tested negative for AVPM-1 antibodies (Morgan et al., 1981) .

Despite serology-based knowledge of these viruses among Antarctic birds, our understanding of their diversity is extremely limited due to the lack of available genomic data. Partial genome sequences of NDV in Adélie penguins has been obtained using HTS

approaches (Thomazelli et al., 2010). Most recently, complete genome sequences of avian paramyxovirus 10 (APMV 10) and three novel avulaviruses (APMV 11, 12, 13) have been determined from rockhopper penguins on the Falkland Islands and gentoo penguins sampled on Kopaitic Island, northern tip of the Antarctic Peninsula (Table 1, Figure 1) (Goraichuk et al., 2017; Neira et al., 2017).

With the use of sled dogs (*Canis familiaris*) during the early Antarctic expeditions, concern of morbillivirus infection among Antarctic pinnipeds drove research in monitoring for this virus in seal populations. Antibodies to canine distemper virus (CDV) have been reported in leopard and crabeater seals around the Antarctic Peninsula (Bengtson et al., 1991) and phocine distemper virus (PDV) in Weddell seals from Vestfold Hills, East Antarctica (McFarlane, 2009). With the exception of crabeater seals, both of these studies revealed low antibody titers against CDV and PDV. Several other studies looking at morbilliviruses in Antarctic seals have failed to detect any antibodies against these viruses (Harder et al., 1991; Osterhaus et al., 1988; Stenvers et al., 1992; Yochem et al., 2009). This may suggest morbilliviruses are not persistent in Antarctic seals or perhaps there are diverse morbilliviruses circulating amongst the pinnipeds that cannot be detected using conventional serology assays but likely to be identified using HTS approaches. Unlike avian paramyxoviruses, no genomic data are available for morbilliviruses from Antarctic seals and therefore impossible to tell if there was a spillover event from the canines to the pinnipeds. Sled dogs are no longer allowed in Antarctica.

3.3 Double stranded RNA viruses

3.3.1 *Birnaviridae*

Viruses in the family *Birnaviridae* have non-enveloped capsids that encapsidate two linear double stranded segments of RNA, each ~2.3-3 kb in length. Four genera have been established in this family: *Avibirnavirus, Aquabirnavirus, Blosnavirus* and *Entomobirnavirus* (Delmas et al., 2011). Infectious bursal disease virus is the only

characterized virus belonging to genus *Avibirnavirus*. Since its initial identification as virus responsible for a highly infectious disease among chickens, infectious bursal disease virus (IBDV) has widely been isolated from other birds in the poultry industry including ducks and turkeys, however, disease has only been identified in chickens. Between 1995-2002 neutralization assays identified high titers of antibodies against IBDV in Adélie penguin at colonies around Mawson and Davis stations, and Terra Nova Bay, East Antarctica; and emperor penguin at the Auster, Amanda and Cape Washington colonies, also in East Antarctica (Table 1, Figure 1) (Gardner et al., 1997; Watts et al., 2009). Highest seroprevalence has been detected among emperor penguin colonies with no difference between sampled locations or years (Watts et al., 2009). South Polar skuas around Vestold Hills and Davis Station, East Antarctica, have also had high titer of antibodies against IBDV (Miller et al., 2008; Watts et al., 2009), however, a significant difference in seroprevalence between sampling periods during 1999-2002 was observed. A low titer and prevalence of antibodies to IBDV has been detected in king penguins around Possession Island, among the Crozet Islands along the APF (Gauthier-Clerc et al., 2002). High-titers of IBDV neutralizing antibodies detected in distant populations of penguins and South Polar skua around Antarctica suggests it is unlikely IBDV was introduced through disposal of chicken products around Mawson Station as previously suggested by Gardner et al. (2007). Given that IBDV infection has been commonly detected in other wild avian populations (Hollmén et al., 2000; Kasanga et al., 2008; Ogawa et al., 1998), this virus may be naturally occurring among Antarctic birds (Watts et al., 2009). It is worth noting, however, that IBDV has yet to be isolated from Antarctic birds despite several studies detecting neutralizing antibodies. Therefore, it is difficult to address any questions about the diversity, evolution or transmission of this virus among Antarctic birds.

3.4 Double stranded DNA viruses

3.4.1 *Adenoviridae*

Adenoviruses are a family of non-enveloped double stranded DNA viruses with a genome length of ~26-45 kb. The diversification of these viruses is thought to have occurred

through several animal hosts including mammals, reptiles, birds, fish and amphibians. The family *Adenoviridae* has been divided into five genera: *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus*, *Ichtadenovirus* (Harrach et al., 2011).

Most adenovirus research has focused on the implications of human-associated adenoviruses, likely due to the known clinical significance in causing respiratory disease and gastroenteritis. However, the first adenoviruses from Antarctic animals have only recently been identified among South Polar skua (*Skua siadenovirus A*) (Park et al., 2012), as well as chinstrap, Adélie and gentoo penguins (*Penguin siadenovirus A*) (Table 2, Figure 1) (Lee et al., 2014; Lee et al., 2016). This provides important insight to monitoring penguin health in Antarctica, as adenoviruses have been known to cause severe disease among animals.

Whole genomes for these adenoviruses were confirmed using HTS approaches and subsequent phylogenetic analyses of the genomic sequences provide support for the classification of the South Polar skua and penguin adenoviruses in the genus *Siadenovirus*. Despite this classification, penguin adenovirus genomes are unique in that they lack a putative sialidase gene that is characteristic of other genomes in this genus (Lee et al., 2016).

It is likely that there exits adenoviruses associated with Antarctic seals based on the fact that adenoviruses has been identified in California sea lion (*Zalophus californianus*), Fur seals (*Arctocephalus* spp.) and South American sea lion (*Otaria flavescens*) (Chiappetta et al., 2017; Cortes-Hinojosa et al., 2016; Cortes-Hinojosa et al., 2015; Goldstein et al., 2011; Inoshima et al., 2013).

3.4.2 *Herpesviridae*

Herpesviridae is a large family of enveloped viruses with a linear, double stranded DNA genome about 120-240 kb in length. This family has been divided into three subfamilies (*Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*). Herpesviruses belonging to two species, based on partial genome sequencing of conserved regions, have been found among pinnipeds in the Northern Hemisphere (Harder et al., 1996): phocid alphaherpesvirus-1 (PhHV-1, species *Phocid alphaherpesvirus 1*) belonging to the *Varicellovirus* genus of the *Alphaherpesvirinae* subfamily and phocid gammaherpesvirus-2 (PhHV-2, *Phocid gammaherpesvirus 2*) belonging to the *Gammaherpesvirinae* subfamily. Both PhHV-1 and PhHV-2 have been identified in several non-Antarctic pinniped species around the world from free-ranging populations as well as captive populations in zoos and aquaria (Bellehumeur et al., 2016; Goldstein et al., 2004; Osterhaus et al., 1985).

Among Antarctic pinnipeds, herpesvirus has not been confirmed by molecular methods, however, several studies over the years have shown high levels of PhHV-1 neutralizing antibodies in Antarctic fur seals among sub-Antarctic islands, and Ross, Weddell and crabeater seals off East Antarctica (Table 1, Figure 1) (Harder et al., 1991; Stenvers et al., 1992; Tryland et al., 2012). Thus, it is highly likely that herpesvirus is widespread and persistent among pinnipeds. However, genomic data is required to confirm this virus among Antarctic pinnipeds as the serological data has only indicated infection of a herpesvirus antigenically similar to PhHV-1.

3.4.3 *Papillomaviridae*

Papillomaviridae is a large family of non- enveloped, circular, double stranded DNA viruses with ~7-8 kb genomes and are known to infect skin, squamous and mucosal epithelial cells. All papillomavirus genomes have a very similar organization that can be

divided into three regions encoding replication associated and regulatory proteins, structural proteins, and a long control region. While research on human papillomaviruses has been extensive due its clinical significance, relatively few studies have looked at non-human papillomaviruses. Papillomaviruses are found in a range of hosts including mammals, birds, reptiles and fish. It is a well-supported hypothesis that they have co-evolved with their hosts given their diversity and host specificity, with supporting phylogenetic analyses that track diversification of papillomaviruses to the evolution of their host (Bernard et al., 2010; de Villiers et al., 2004).

Two novel papillomavirus, Pygoscelis adeliae papillomavirus 1, -2 (PaPV1, -2), was recently identified in Antarctica from feces and cloacal swab of Adélie penguins at Cape Crozier, Ross Island (Table 2, Figure 1) using a HTS-informed approach (Van Doorslaer et al., 2017; Varsani et al., 2014). PaPV1 and -2 are related to other avian papillomaviruses, PaPV1 has been assigned to the genus *Treisepsilonpapillomavirus* whereas PaPV2 is currently unclassified and shares ~64% genome-wide pairwise identity with PaPV1. These PaPVs are the first papillomaviruses to be discovered in Antarctic animals and are part of the few known avian papillomaviruses.

3.4.4 *Polyomaviridae*

Polyomaviruses represent a family of non-enveloped, circular, double-stranded DNA viruses with a genome length of 5-6 kb, and infect a range of hosts including mammals, birds, reptiles and fish. This family has four genera: *Alphapolyomavirus*, *Betapolyomavirus*, *Deltapolyomavirus*, and *Gammapolyomavirus* with three species unassigned to any of these (Moens et al., 2017; Polyomaviridae Study Group of the International Committee on Taxonomy of et al., 2016). Phylogenetic analyses have shown that avian polyomaviruses cluster together and have been classified under the genus *Gammapolyomavirus*. Avian polyomaviruses are known to cause inflammatory disease in birds, and can lead to disease of the skin and feathers and mortality in some species. The first polyomavirus identified in Antarctica was found in the feces of Adélie penguins

at Cape Royds, Ross Island (Varsani et al., 2015) using a HTS-informed approach. Analysis of this genome shows that it falls in the avian polyomavirus lineage, representing a novel species (*Pygoscelis adeliae polyomavirus 1*).

Following this, two other polyomaviruses have been identified from Antarctic animals: a polyomavirus from the stomach of a sharp-spined notothen (*Trematomus pennellii*) (Buck et al., 2016) and most recently from the kidney of a Weddell seal, both sampled in the Ross Sea (Table 2, Figure 1) (Varsani et al., 2017). The sharp-spined notothen polyomavirus is one of the three polyomaviruses to be identified associated with fish and all three were identified using HTS approaches (Buck et al., 2016).

Polyomavirus sequences have been identified in three other pinniped species: once in a captive Hawaiian monk seal (*Neomonachus schauinslandi*) (Cortes-Hinojosa et al., 2016), in the placenta of one northern fur seal (*Callorhinus ursinus*) (Duncan et al., 2013) from Alaska and a stranded free-ranging California sea lion (*Zalophus californianus*) (Colegrove et al., 2010). However, until recently the genome of California sea lion polyomavirus (CSLPyV) was the only confirmed pinniped polyomavirus. The recently identified Weddell seal polyomavirus is has been proposed to be classified as the species *Leptonychotes weddellii polyomavirus 1* (https://talk.ictvonline.org/files/proposals/animal_dna_viruses_and_retroviruses/m/animal_dna_ec_approved/6941).

3.4.5 *Poxviridae*

Poxviruses are a diverse family of double-stranded DNA viruses with a wide host range among vertebrates and arthropods (Skinner et al., 2011). Poxviruses have been extensively studied for their clinical significance in causing highly pathogenic disease among humans and other animals. While sealpox has yet to be formally classified, studies have identified this virus in populations of harbor seals (*Phoca vitulina*) (Muller et al.,

2003), gray seals (*Halichoerus grypus*) (Nettleton et al., 1995), Stellar sea lions (*Eumetopias jubatus*), spotted seals (*Phoca largha*) (Bracht et al., 2006), and California sea lions (Nollens et al., 2006), causing severe proliferative lesions on the bodies of infected individuals. Partial sequencing has indicated seal poxvirus falls under the parapoxvirus family of which only four species have been classified.

The only case of poxvirus in Antarctica known to date has been the isolation and detection from a skin lesion of a deceased Weddell seal in Queen Maud Land, East Antarctica (Table 2, Figure 1) (Tryland et al., 2005). Other Weddell seals in the area were analyzed for seal poxvirus, however, all individuals were negative, suggesting poxviruses may not prevalent in this population. Partial sequencing of the Weddell seal parapoxvirus shows it is closely related to harbor and grey seal poxviruses (Tryland et al., 2005).

Recently a seal parapoxvirus was sequenced using HTS from a skin lesion of a grey seal from the Baltic Sea (Gunther et al., 2017). While poxviruses have been identified in several avian species, very little is known about their diversity and host range. Using HTS technology a novel avipoxvirus genome has been sequenced from an African penguin (*Spheniscus demersus*) (Offerman et al., 2014). It is highly likely that poxviruses will also be recovered from Antarctic penguins through HTS.

3.5 Single stranded DNA viruses

3.5.1 *Anelloviridae*

Viruses in the family *Anelloviridae* are non-enveloped, circular single stranded DNA viruses with a genome length of about 2-3.9 kb (Biagini et al., 2011). These viruses have high sequence variability and are highly prevalent in the environment. Despite their ubiquitous nature, the significance of infection and pathogenicity remains unknown. While research has focused on their diversity and significance in humans, anelloviruses have been identified in non-human primates, domesticated animals, rodents and recently

in marine mammals. Analysis of lung tissue from a captive California sea lion showing signs of respiratory disease led to discovery of the first anellovirus among pinnipeds, Zalophus californianus anellovirus (ZcAV) (Simpson et al., 2009). Since then several novel anellovirus genomes have been recovered in harbor seals (Bodewes et al., 2015; Bodewes et al., 2013). In lung samples of deceased harbor seals along the North American Pacific coast anelloviruses were identified over multiple years demonstrating the persistence of this infection in the population (Ng et al., 2011). Analyses of sub-Antarctic (*Arctocephalus tropicalis*) and South American fur seal (*A. australis*) feces also led to the identification of anellovirus sequences (Kluge et al., 2016).

Anelloviruses circulating in the Antarctic ecosystem have recently been shown following detection by HTS and using pairs of abutting primers in the recovery of 152 genomes from vaginal, nasal and faecal samples of Weddell seals in the Ross Sea during the 2014-2015 summer (Fahsbender et al., 2017). Analyses identified two novel anelloviruses, torque teno Leptonychotes weddellii virus (TTLwV-1, TTLwV-2). TTLwV-1 was additionally identified in South Polar skua faecal samples and it was thought that this was as a result of skuas feeding on the placenta and dead carcasses of Weddell seals in the area (Fahsbender et al., 2017).

4. *Potential vectors of viruses associated with Antarctic wildlife*

Both ecto- and endoparasites have been reported among Antarctic animals. While neither appear to be detrimental to animal health, these organisms may play a significant role as vectors of viruses. Ticks, mites and lice commonly parasitize seals, penguins and other Antarctic birds (Gauthier-Clerc et al., 1998; González-Acuña et al., 2013; McFarlane, 1996). Flaviviruses, orbiviruses, phleboviruses, and nairoviruses have been isolated from seabird ticks (*Ixodes uriae*) associated with king, rockhopper and royal penguins on Macquarie Island (Major et al., 2009). A novel alphavirus has also been isolated and partially sequenced from lice associated with southern elephant seals of Macquarie Island and the high seroprevalence in the southern elephant seal population showed to this virus

strongly suggests its transmission by lice (La Linn et al., 2001). Gastrointestinal parasites, particularly cestode and nematode species, are commonly found in Antarctic seals and penguins. Penguins tend to have a low diversity of parasites and similar profiles have been identified among penguins of the same genus (Diaz et al., 2016; Diaz et al., 2013; Fonteneau et al., 2011; Kleinertz et al., 2014; Vidal et al., 2012). Of the Antarctic seals, gastrointestinal parasites are most prevalent among Weddell and leopard seals. Given that helminth parasites are strongly associated with the diet of the host they infect, this likely explains the higher abundance of parasites among Weddell and leopard seals compared to other Antarctic seals (McFarlane et al., 2009). The potential for endoparasites to transmit viruses to their host has been demonstrated by two genera of plant viruses, nepoviruses and tobraviruses, transmitted by nematodes (Hull, 2014). While our knowledge of parasites in Antarctic animals remains extremely limited and research in this area has been sporadic, developments in molecular technology will undoubtedly have a strong impact toward revealing relationships between organisms and the movement of viruses in the environment.

Recently, Antarctic penguins have been showing signs of disease of unknown pathology, e.g. unexplained incursions of feather loss in Adélie penguins (Grimaldi et al., 2015) in the Ross Sea (2011 – 2012) but not the years before or after (personal observation). Furthermore, in 2014 observations of an Adélie penguin colony at Hope Bay, Antarctica identified two chicks showing patches without feathers in two sub colonies (Barbosa et al., 2014). Beak and feather disease virus (family *Circoviridae*) infection in certain psittacines causes feather abnormalities and loss (Pass and Perry, 1984) and hence there is a likelihood that feather loss observed in penguins may be attributed to an unknown circovirus-like agent.

5. *Concluding remarks and future directions*

Over the last ten years, viral metagenomics has led to a dramatic increase in viral discovery from various environmental and animal samples, for example, Shi et al. (2016)

identified ~1400 novel RNA viruses (from over 200 invertebrate species), Brum et al. (2015) identified ~5500 distinct dsDNA virus populations (from 43 surface ocean sites worldwide as part of the Tara Oceans expedition) and Paez-Espino et al. (2016) identified ~120000 partial viral genomes sequences (from ~3000 geographically diverse samples) using HTS. This together with other studies that have identified large datasets of novel viruses using HTS (e.g. (Dayaram et al., 2016; Labonte and Suttle, 2013; Rosario et al., 2015) has shown: 1) the HTS enabled identification of large numbers of previously unknown viruses; 2) we have barely scratched the surface of the viral sequence space and thus their diversity; 3) new taxa will need to be created to classify viruses at a rapid rate to match the pace of virus discovery. All this has opened up discussions on viral classification based on sequence data, either derived from Sanger sequencing or HTS, and lead to a consensus statement by Simmonds et al. (2017a) to incorporate these into current viral taxonomy.

HTS has been used to a large extent in Antarctic environmental virology to study soil (Adriaenssens et al., 2017; Zablocki et al., 2014), lake (Aguirre de Carcer et al., 2016; Lopez-Bueno et al., 2015; Lopez-Bueno et al., 2009; Yau et al., 2011) and marine (Brum et al., 2017; Miranda et al., 2016) viral ecology. Novel viral genomes from various soils and lake samples (Dziewit and Radlinska, 2016; Kerepesi and Grolmusz, 2017; Meiring et al., 2012; Swanson et al., 2012; Zawar-Reza et al., 2014) have been determined using HTS approaches. In contrast, relatively little is known about viruses associated with Antarctic animals and the associated virus ecology despite the advent of HTS. This perhaps can be attributed to the difficulty in accessing / obtaining animal samples and longitudinal sampling for viral ecology studies. Nonetheless, various studies have used HTS to identify viruses associated with Antarctic animals (Table 2, Figure 2) and we anticipate that the next decade will see a dramatic increase in virology activity and to some extent viral ecology studies of Antarctic animals. Furthermore, it is highly like that large numbers of diverse viruses will be identified using HTS. As sequencing technologies improve, there may be possibility of in-field identification of animal viral pathogens in Antarctica, e.g. using Oxford Nanopore Sequencer (ONS). ONS has been used for metagenomic studies of microbial mats from three lakes in the Antarctic dry

valleys (Lakes Fryxell, Lake Vanda and Lake Vida) by Johnson et al. (2017) demonstrating its use in Antarctic field conditions and remote laboratories.

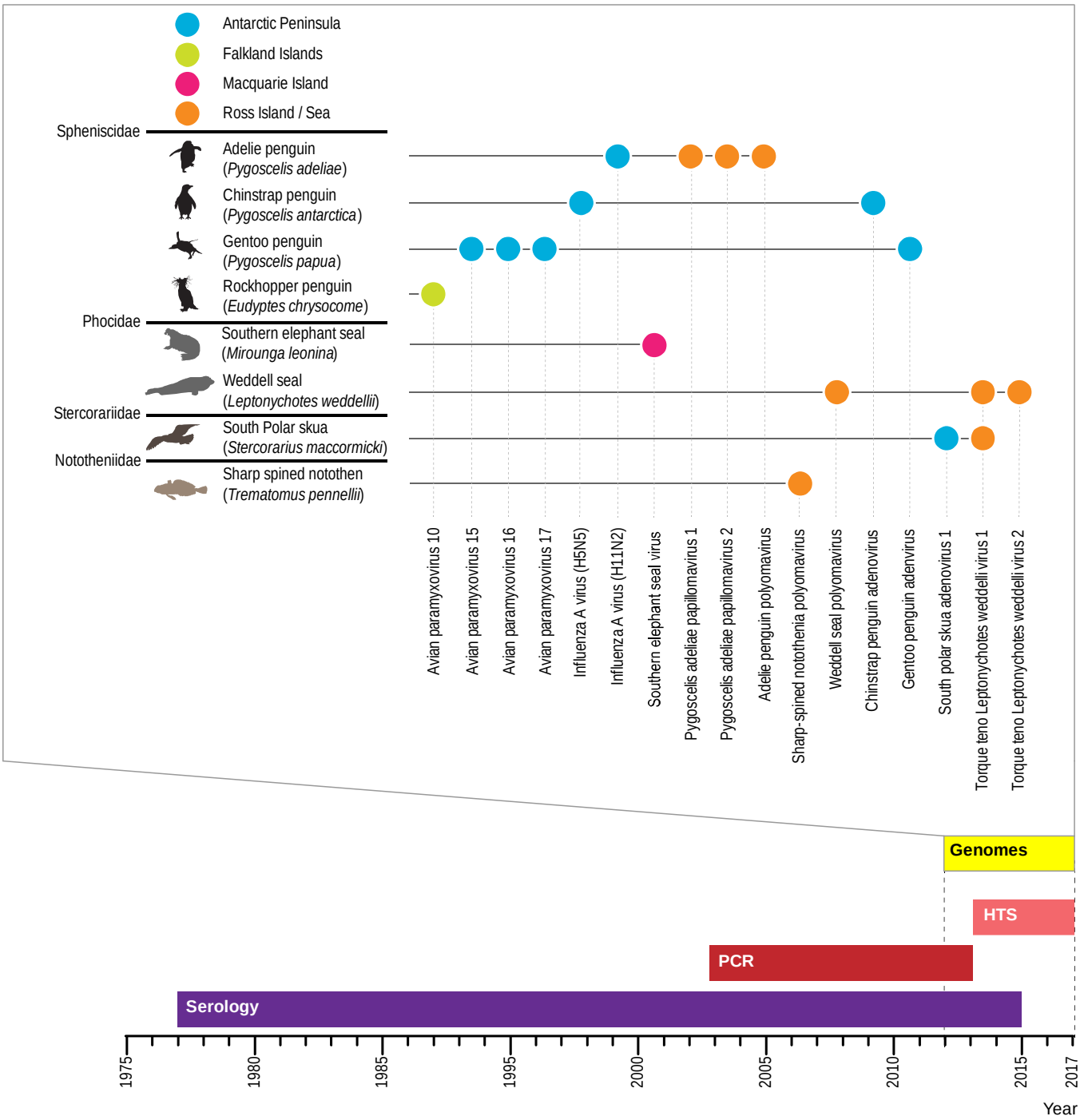


Figure 2: Timeline,1975-2017 (present), showing the periods of serology- (purple), PCR- (red) and HTS-based (light red) approaches for viral identification. From 2012 onwards, viral genomes are determined by PCR-, Sanger- and HTS-based approaches. Top panel summarizes the determination of complete genomes by either Sanger sequencing and / or HTS from associated Antarctic animals. Colored circles indicating where they were found: Antarctic Peninsula (dark blue), Falkland Islands (green), Macquarie Island (pink) and Ross Island / sea (orange).

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Diverse papillomaviruses identified in Weddell seals

Abstract

Papillomaviridae is a diverse family of circular, double-stranded DNA (dsDNA) viruses that infect a broad range of mammalian, avian and fish hosts. While papillomaviruses have been characterised most extensively in humans, the study of non-human papillomaviruses have greatly contributed to our understanding of their pathogenicity and evolution. Using high throughput sequencing approaches, we identified seven novel papillomaviruses from vaginal swabs collected from 81 adult female Weddell seals (*Leptonychotes weddellii*) in the Ross Sea of Antarctica between 2014-2017. These seven papillomavirus genomes were amplified from seven individual seals, and six of the seven genomes represented novel species with distinct evolutionary lineages. This highlights the diversity of papillomaviruses among the relatively small number of Weddell seals samples tested. Viruses associated with large vertebrates are poorly studied in Antarctica, this study adds information about papillomaviruses associated with Weddell seals and contributes to our understanding of papillomavirus evolutionary history.

1. Introduction

Papillomaviridae is a family of circular double stranded DNA viruses that have ~ 7-8kb genomes. There are >350 distinct papillomavirus types that infect skin, squamous and mucosal epithelial cells (Rector and Van Ranst, 2013) in a wide range of hosts including mammals, birds, reptiles and fish. Papillomaviruses have a genome organization that can be divided into three major regions; early, late and a long control region (LCR) which is involved in viral replication (Doorbar, 2005). Upon infection, the early genes (E1, E2 and

E4) are expressed and involved in DNA replication and transcription regulation. Most papillomaviruses encode at least one additional early protein (E5, E6, or E7) involved in manipulating the cellular environment. Expression of these proteins compromises the regulation processes of the cell cycle and leads to proliferation of infected cells. In certain papillomaviruses, these proteins are oncogenic in their hosts (Dyson et al., 1989; Munger et al., 1992; Munger et al., 1989; Yew and Berk, 1992), likely in part due to their ability to inactivate p53 and pRb proteins that have an essential role in modulating the cell cycle. The viral late region includes two structural proteins that form the viral capsid (L1 and L2) and are expressed later once infected cells have proliferated to the squamous and mucosal epithelial layer. The LCR is involved in viral replication (Doorbar, 2005).

Most papillomaviruses identified to date have been associated with humans. The human papillomaviruses (HPVs) are classified in five genera *Alpha-*, *Beta-*, *Mu-*, *Nu-*, and *Gammapapillomavirus.* Non-human animal papillomaviruses have been characterised in hosts across 18 taxonomic orders (López-Bueno et al., 2016; Rector and Van Ranst, 2013). Consistent with the co-evolution hypothesis, phylogenetic analyses have shown the divergence of mammalian papillomaviruses from avian and reptile papillomaviruses (Van Doorslaer et al., 2017b). Better understanding of papillomavirus diversity and their evolutionary history will help to elucidate impacts of the virus on the health of wild animal populations. This has important implications for wildlife conservation management.

Just as viral diversity is relatively better understood in humans than in other species, it is also better understood in regions where human presence is long-established. In more remote regions such as polar ecosystems, little is known about viruses and associated diseases. Yet these areas are just those that are predicted to change most and for understanding the polar disease ecology it essential to collect base line data on viruses and other microbes circulating within these ecosystems. For example, very little is known about viruses circulating amongst Antarctic animals (Smeele et al. (2018), but there is evidence that endemic Antarctic species are infected with a similar viral diversity to that in other regions. The handful of viral genomes from Antarctic animals that have been

identified include those that belong to the viral families *Adenoviridae* (Lee et al., 2014; Lee et al., 2016; Park et al., 2012), *Anelloviridae* (Fahsbender et al., 2017), *Orthomyxoviridae* (Hurt et al., 2016; Hurt et al., 2014), *Papillomaviridae* (Van Doorslaer et al., 2017b; Varsani et al., 2014), *Paramyxoviridae* (Goraichuk et al., 2017; Miller et al., 2010; Neira et al., 2017; Thomazelli et al., 2010), *Polyomaviridae* (Buck et al., 2016; Varsani et al., 2017; Varsani et al., 2015), *Poxviridae* (Tryland et al., 2005) and *Togavirdae* (Forrester et al., 2012; La Linn et al., 2001).

The four Antarctic seals, Weddell, leopard (*Hydrurga leptonyx*), crabeater (*Lobodon carcinophaga*) and Ross (*Ommatophoca rossii*), are classified in the taxonomic tribe Lobodontini within the Phocidae family. Pinnipeds form a clade within the order Carnivora and diverged most recently from the Ursidae family. Within the carnivore order, pinnipeds diverged after the split of Feliformia and Caniformia, and therefore, seals are also closely related to canines and mustelids (Bininda-Emonds et al., 1999; Higdon et al., 2007). Weddell seals are the most southerly distributed pinniped, breeding on the fast ice of Antarctica. They remain at high-latitudes year-round by maintaining breathing holes along tidal cracks in the fast ice throughout winter months, and consume a diverse diet (Burns et al., 1998; Castellini et al., 1991; Stirling, 1969). Building on the first identification of a papillomavirus in pinnipeds (ZcPV1) from California sea lions (*Zalophus californianus*) (Rivera et al., 2012) this study aimed to assess whether the southernmost mammal, living in the isolated and unique polar habitat of the Ross Sea, Antarctica, is associated with unique and diverse papillomaviruses.

2. Materials and methods

2.1 Sampling and sample processing

Across three Antarctic field seasons, vaginal swabs were taken from a total of 81 (2014/2015, n=25: 2015/2016, n=29; 2016/2017, n=27) individual adult female Weddell seals (Leptonychotes weddellii). All applicable international, national, and institutional guidelines for the care and use of animals were followed during sampling. The vaginal

swabs were stored in UTM™ Viral Transport Media (Copan, USA) at 4□C for up to 6 months prior to analysis. 1ml of the transport media was filtered through a 0.2µm syringe filter for each sample and 200 µl of the filtrate was used for viral DNA extraction using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, USA). Viral circular DNA molecules subsequently were amplified using rolling-circle amplification (RCA) using the TempliPhi™ kit (GE Healthcare, USA).

2.2 High throughput sequencing and sequence analysis

The RCA products (5 µl from each sample) were pooled for each season. The three pooled RCA products were used to prepare 2×100bp libraries and these were sequenced on an Illumina HiSeq4000 at Macrogen Inc. (South Korea). The resulting paired-end reads were de novo assembled using ABySS v2.02 (kmer=64) (Simpson et al., 2009). BLASTx (Altschul et al., 1990) analysis of assembled contigs >2000 nts revealed seven contigs that had similarities to papillomavirus sequences. Abutting primers (Table 1) were designed based on each PV-like de novo assembled viral contig to recover the full genomes from individual samples. Amplification of the papillomavirus-like molecules was carried out using KAPA Hifi Hotstart DNA polymerase (Kapa Biosystems, USA), the abutting primers, RCA product as template (0.5 µl) with the following polymerase chain reaction (PCR) protocol on an Eppendorf Mastercycler: initial denaturation at 95˚C for 3 minutes, then 30 cycles at 95˚C for 30 seconds, 60˚C for 30 seconds, 72˚C for 8 minutes and a final extension at 72˚C for 8 minutes. The amplicons were resolved on a 0.7% agarose gel, gel purified and cloned into pJET1.2 plasmid (ThermoFisher, USA). The resulting recombinant plasmids were Sanger sequenced at Macrogen Inc. (South Korea) by primer walking. The Sanger sequenced contigs were assembled using DNA-baser v.4 (Heracle BioSoft S.R.L).

Representative annotated papillomavirus genomes (n=352) sequences were downloaded from the PaVE database (Van Doorslaer et al., 2017a; Van Doorslaer et al., 2013). From the 352 annotated sequences downloaded from PaVE and the seven papillomaviruses

genomes recovered in this study, the L1 gene, and E1, E2 and L1 protein sequences were extracted. Two datasets were created, one of aligned L1 gene sequences and the other of concatenated aligned E1, E2 and L1 protein sequences. All alignments were carried out using MAFFT (Katoh et al., 2002).

The aligned L1 gene sequences were used to infer a maximum likelihood phylogenetic tree using PhyML 3.0 (Guindon et al., 2010) with GTR+I+G4 as best fit model determine using jModelTest (Darriba et al., 2012). A maximum likelihood phylogenetic tree for the concatenated aligned E1, E2 and L1 proteins was inferred using PhyML 3.0 (Guindon et al., 2010) and LG+F+I+G4 model determined as best fit model for E1, E2 and L1 partitions determined using PartitionFinder 2 (Lanfear et al., 2017). Branches with <0.75 aLRT branch support (Anisimova and Gascuel, 2006) were collapsed using TreeGraph 2 (Stover and Muller, 2010). The phylogenetic trees were visualised and edited using iTOL v3 (Letunic and Bork, 2016). The pairwise identities of the full genomes and L1 gene were determined using SDT v1.2 (Muhire et al., 2014). The Carnivora host phylogeny was inferred with TimeTree (http://www.timetree.org/) (Kumar et al., 2017).

3. Results and discussion

3.1 Papillomaviruses associated with Weddell seals

We identified and recovered seven papillomavirus genomes that range in size from 7392 to 7836 nts from individual Weddell seals (age 10-19 at the time of sampling) from the three seasons (Figure 1; Table 1). Seals were deemed clinically healthy at the time of sampling, and no lesions or papillomas were noted.

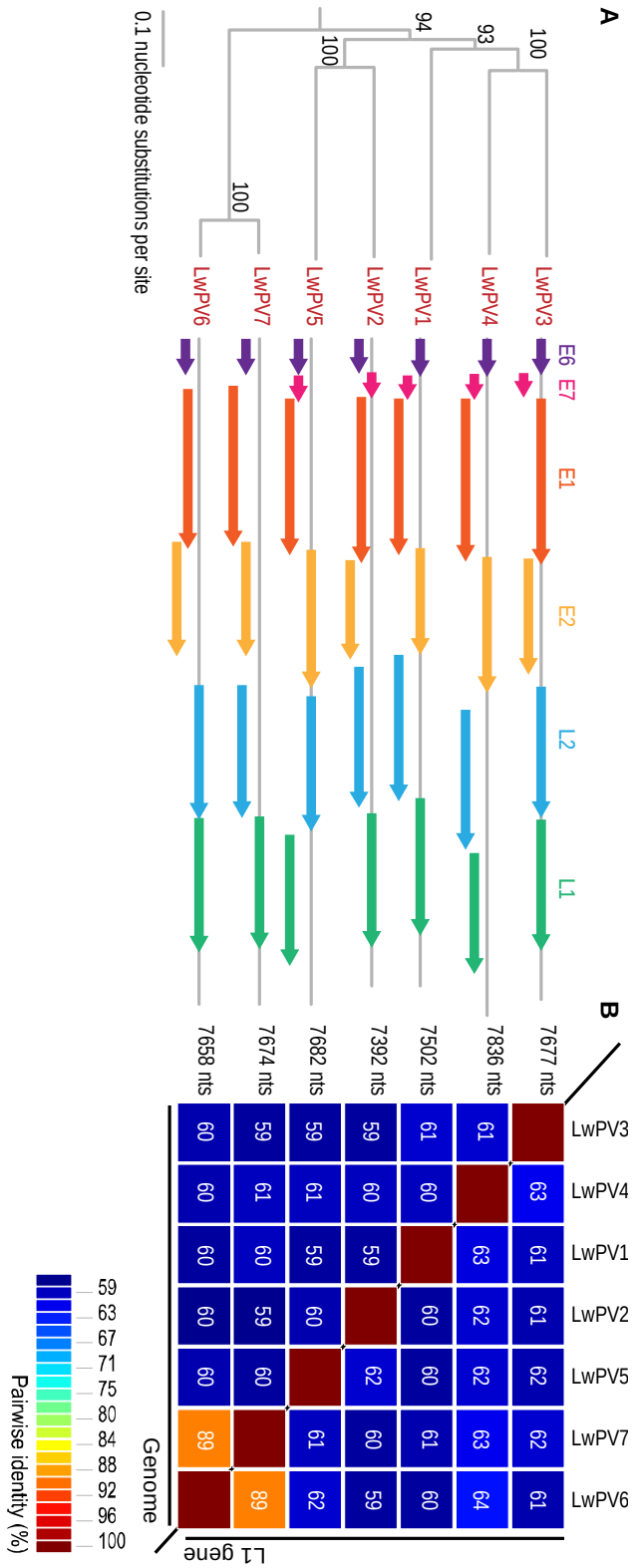

Figure 1: (A) Neighbor joining phylogenetic tree of the tree inferred from the aligned genome sequences of LwPV1-7 with the genome organisation for each genotype showing E6, E7, E2, L2 and L1 ORFs coupled with genome size. (b) Pairwise identity plot with percentage pairwise identities provided in colour box for the genome and the L1 nucleotide sequences.

Table 1: Sample data for LwPV1-7 providing GenBank accession number (MG571089-MG571095), season the LwPV was identified, specimen number (SPENO) for individual seal in which the respective virus was identified, age of individual at time of sampling, date sample was taken and abutting primer pair used to recover genome.

Name	Accession #	Season	SPENO	Age at sampling	Sampling date	Primers
Leptonychotes weddellii papillomavirus 1	MG571090	2014/2015	13241	16	11/19/2014	F: 5'-GCCTTATACTCATCAGGCTTATATGGATTTTGGG-3' R: 5'-CTACCTAAGGCATACAGAGGAATATGACATTGCAT-3'
Leptonychotes weddellii papillomavirus 2	MG571089	2014/2015	11445	19	1/18/2015	F: 5'-TAATTTCAGAAACCTGTGATGCTGGAAGTGTTTG-3' R: 5'-ATACTAAGCATGCTATTATGATGAAGTTGGTTTT-3'
Leptonychotes weddellii papillomavirus 3	MG571093	2015/2016	17181	10	11/30/2015	F: 5'-TATCCCTTTAATGATGAAGGACAGCCCACATATCT-3' R: 5'-CTCATTTTTAAAGGTAAAGCACTGCAGTCTGCTG-3'
Leptonychotes weddellii papillomavirus 4	MG571095	2014/2015	12091	18	1/28/2015	F: 5'-AAAGTGTTGCCTTCCACTTTATTTACAACATCATC-3' R: 5'-TGCAGATAGATTTTTAAAATGGGCAAGCTCTTTTC-3'
Leptonychotes weddellii papillomavirus 5	MG571094	2016/2017	12975	18	11/24/2016	F: 5'-TAAACAGTGACACACAGCTGTTTAACAGGCCTTTT-3' R: 5'-CTAGAGAACCGCTGGGAGTTCCATAGTAGATAGAA-3'
Leptonychotes weddellii papillomavirus 6	MG571091	2016/2017	17495	10	12/8/2016	F: 5'-CATATCCAATAAAGTCAGATGATTCAGGAGGTAGC-3' R: 5'-CTGTGTATAGTGACTTCTATTATGACCCTAGCCTT-3'
Leptonychotes weddellii papillomavirus 7	MG571092	2016/2017	17629	10	1/19/2017	F: 5'-GGCTATACTAACCTGATTTAGTATCCATTTTGGCC-3' R: 5'-CACATATCACAGGACAGTACACCATTTGAACTATC-3'

The L1 gene is relatively conserved among papillomaviruses and is the current basis for classification within this family, with the International Committee on Taxonomy of Viruses (ICTV) recommending that L1 gene sequences that share <90% pairwise identity with those previously classified should be considered unique papillomavirus types. The L1 sequences of the seven recovered papillomaviruses from Weddell seals share <89% pairwise identity (Figure 1). When compared to all other papillomavirus L1 sequences, the ones from this study share <67% pairwise identity. Thus, the seven papillomaviruses are all new types and have been named *Leptonychotes weddellii* papillomavirus 1-7 (LwPV1-7). A summary of the protein sequence similarities as determined by BLASTp (Altschul et al., 1990) is provided in Figure 2. LwPV6 and -7 which are most closely related sharing 89% genome-wide identity both lack an E7 open reading frame (ORF) (Figure 1). Both the E6 and E7 protein sequences of LwPV1-5 contain canonical Zinc binding motifs ($CX_2CX_{21–23}CX_2C$ metal-binding motif) in both the E6 and E7 protein sequences. In the E7 sequences of three LwPVs (LwPV1, -3, and -4) we identified the conserved pRB LxCxE binding motif (Figure 2). LwPV2 and LwPV5 have a slightly modified LxSxE motif. In other papillomaviruses, similarly belonging to the *Gamma-* and *Taupapillomaviridae,* this modified motif is not involved in binding to and degradation of pRb (Wang et al., 2010).

A

LwPV	Protein	PV	Genus	E-Value	% Pairwise Identity	% Query coverage
LwPV1	E1	PlpPV1	Lambda	0	61.5	100.0
	E2	CPV6	Lambda	8.07E-133	50.7	100.0
	E6	AmPV4	Lambda	3.33E-43	49.6	95.9
	E7	AmPV4	Lambda	7.62E-25	49.5	100.0
	L1	AmPV4	Lambda	0	63.9	96.8
	L2	CPV6	Lambda	5.16E-159	49.0	97.9
LwPV2	E1	HPV4	Gamma	0	50.2	98.0
	E2	VvPV1	Treiseta	6.87E-103	42.3	100.0
	E6	HPV178	Gamma	4.52E-22	39.5	84.3
	E7	HPV153	Gamma	5.47E-30	49.5	99.0
	L1	HPV4	Gamma	0	56.5	99.6
	L2	HPV180	Gamma	1.06E-146	48.5	98.5
LwPV3	E1	AmPV3	Dyonu	0	55.5	100.0
	E2	ZcPV1	Dyonu	1.26E-121	48.4	99.8
	E6	ZcPV1	Dyonu	9.57E-49	58.5	92.5
	E7	CPV12	Chi	2.08E-29	48.0	100.0
	L1	AmPV3	Dyonu	0	63.3	100.0
	L2	ZcPV1	Dyonu	1.29E-152	57.0	100.0
LwPV4	E1	CPV15	Chi	0	54.5	100.0
	E2	CPV12	Chi	3.97E-82	37.2	98.5
	E6	CePV2	Xi	8.60E-34	44.4	93.7
	E7	LwPV3	[Dyonu]	2.03E-22	50.0	96.7
	E7	SscPV3	Dyoomikron	1.32E-20	40.4	97.8
	L1	FcaPV2	Dyotheta	0	63.4	99.8
	L2	MfPV2	Beta	2.89E-91	38.9	100.0
LwPV5	E1	CPV2	Tau	0	57.6	98.7
	E2	CPV7	Tau	6.66E-71	56.2	38.5
	E6	MpPV1	Tau	4.71E-36	41.3	98.6
	E7	MpPV1	Tau	3.88E-30	50.0	100.0
	L1	CPV17	Tau	0	57.9	99.2
	L2	CPV2	Tau	2.59E-120	46.0	98.6
LwPV6	E1	LwPV7	[Omega]	0	92.9	100.0
	E1	AmPV1	Omega	0	62.2	100.0
	E2	LwPV7	[Omega]	0	81.4	100.0
	E2	AmPV1	Omega	2.03E-77	56.5	48.5
	E6	LwPV7	[Omega]	3.47E-87	84.3	100.0
	E6	AmPV1	Omega	1.19E-40	48.9	99.3
	L1	LwPV7	[Omega]	0	92.3	100.0
	L1	AmPV1	Omega	0	68.6	100.0
	L2	LwPV7	[Omega]	0	87.9	100.0
	L2	AmPV1	Omega	3.48E-125	46.7	99.6
LwPV7	E1	LwPV6	[Omega]	0	92.3	100.0
	E1	AmPV1	Omega	0	61.5	100.0
	E2	LwPV6	[Omega]	0	80.5	100.0
	E2	UmPV1	Omega	2.42E-94	43.4	99.1
	E6	LwPV6	[Omega]	3.47E-87	84.3	100.0
	E6	AmPV1	Omega	1.24E-39	48.2	99.3
	L1	LwPV6	[Omega]	0	92.3	100.0
	L1	AmPV1	Omega	0	67.5	100.0
	L2	LwPV6	[Omega]	0	87.9	100.0
	L2	AmPV1	Omega	9.32E-133	48.0	99.6

California sea lion (*Zalophus californianus*)

Weddell seal (*Leptonychotes weddellii*)

B

LwPV1 CKFCHRYLTTLEKLRFDAAPFQLIWKEGNVYGCCQTC
LwPV2 CVFCSRPLDEQDKKNFDKKALKLIRKRGRVFGACGLC
LwPV3 CVFCKKYLNFQERCSFDEKNLQLSWKDSRAYGCCSHC
LwPV4 CLFCQTFLDFASLCHQVYADLQLVWKGREVHGSCVRC
LwPV5 CVFCGAPMTWPDKVAFENKCLSVVWCRERFFGACVSC
LwPV6 CVYCMCALTRRELILFDYRTLFLVWRKGIPYAVCDPC
LwPV7 CVYCLCQLTRRELIIFDYRDLCLVWRKGIPYGVCDPC

Zinc binding mofit

C

LwPV1 CSFCLHTLTYWEKKENKFDSALF-LVRGKVRGTCDLC
LwPV2 CRWCMTLLTVEEKQAVFDRGEDLSLSRSQWRGNCENC
LwPV3 CFKCLRKLSCTEVCAALGGGQYFDLVRGRWKAQCKFC
LwPV4 CLWCLRRLSLTEKIDLSAFRHPVHKVRGRWRALCRVC
LwPV5 CMYCLAQLTVLEKIACAVNERPFVLVRKLFRNTCSEC
LwPV6 CAGCCKYLGEEEKLLMVEENRRVHQIGDKWRGVCIRC
LwPV7 CAGCCKYLGEEEKLLMAEEGRRVHQIGDMWRGVCIRC

Zinc binding mofit

D

LwPV1 LVCYE CGICKRTVRCVC-LAEESDVRIFQELLCGTFCFVCQSC
LwPV2 LLSGE CVSCGASLRLAV-VATNPAIRGLQTLLLGDLSILCAQC
LwPV3 LWCYE CGFCGRGIELVV-LSSRVAIRTLQHLLLENLDIVCPDC
LwPV4 LYCNE CGHCDRPVRLVI-LASRDQVRVLHRLLLEGLCFVCPYC
LwPV5 LLSGE CGGCHTPVRLCIQVVHRTLVHKFEELLLEGLDIVCATC

Lx[C/S]xE

Zinc binding mofit

Figure 2: (A) BLASTp results for each of the proteins encoded by LwPV1-7 with Weddell seal papillomaviruses highlighted in red and California sea lion papillomavirus 1 (ZcPV1) in pink. Protein sequence of first (B) and second (C) zinc binding motif ($CX_2CX_{21–23}CX_2C$) present in L6 ORF of LwPV1-7. (D) Protein sequence of pRB binding motif (Lx[C/S]xE) and zinc binding motif in E7 ORF of LwPV1-5.

Sequences that share <60% L1 nucleotide sequence identity are classified into different genera, while novel species isolates have 60-70% sequence identity, while types differ by at least 10% sequence identity (De Villiers et al., 2004). Based on pairwise L1 nucleotide sequence comparisons and L1 phylogenetic tree support (Figure 3), LwPV1-5 are likely members of new species within existing genera (LwPV1, *Lambdapapillomavirus*; LwPV2, *Trisetapapimmomavirus*; LwPV3, *Dyonupapillomavirus*; LwPV4, *Dyothetapapillomavirus*; LwPV5, *Taupapillomavirus*). Furthermore, it is apparent that LwPV6 and -7 share ~68-71 % pairwise identity and cluster with *Ailuropoda melanoleuca* papillomavirus (AmPV) 1-2 and *Ursus maritimus* papillomavirus 1 (UmPV1) in the *Omegapapillomavirus* genus (Figure 3). Thus, LwPV6 and -7 are likely to be assigned to a new species in the genus *Omegapapillomavirus*.

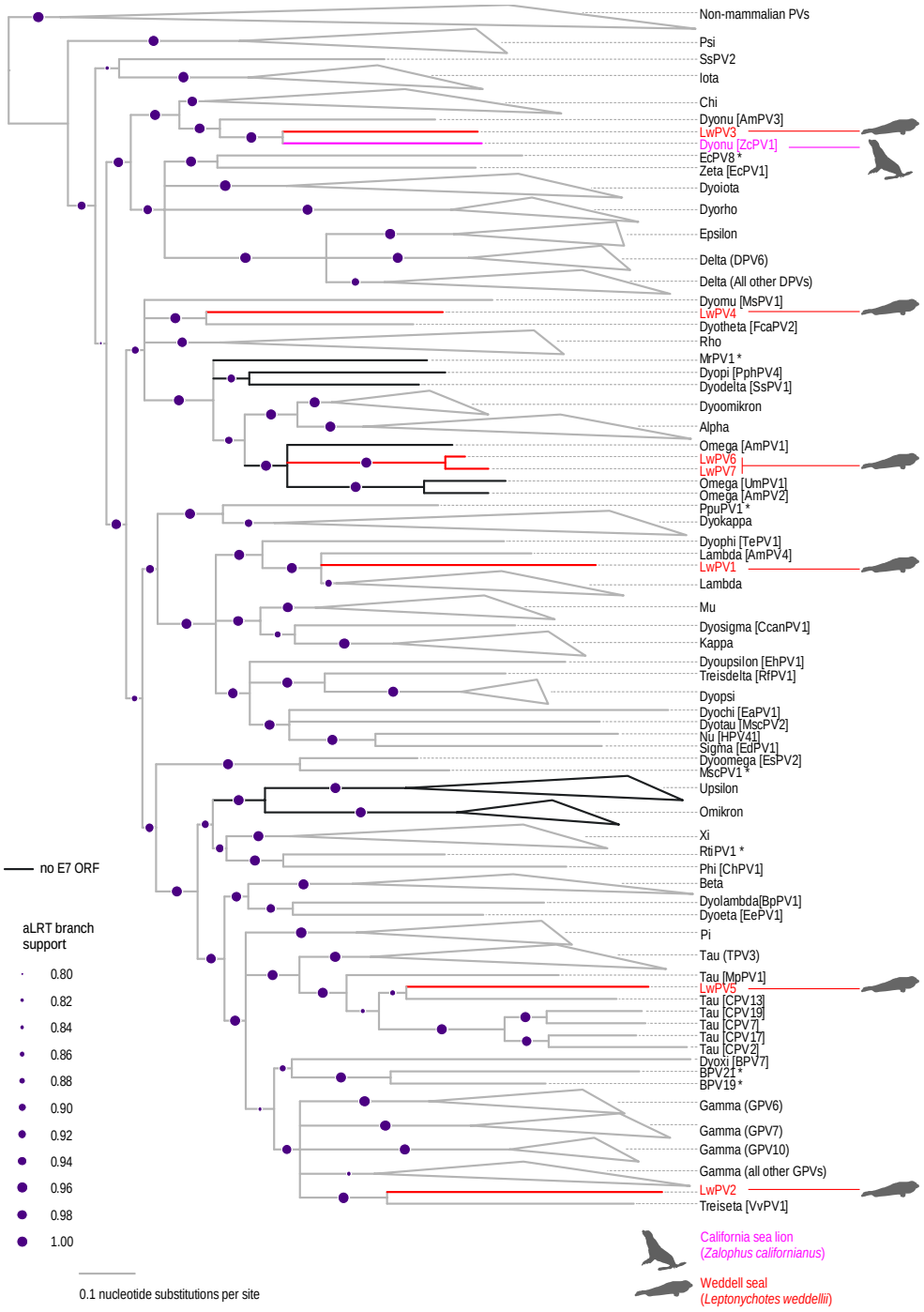

Figure 3: Maximum likelihood tree based on L1 nucleotide sequence using the best-fit model GTR+I+G4. LwPV1-7 highlighted in red, with red branch showing lineage, California sea lion papillomavirus ZcPV1 highlighted in pink, with pink branch showing lineage. Branches in black indicated lineages absent of E7 gene. Branch support values indicated in blue circle size gradient. Branches with < 0.75 aLRT branch support were collapsed. Papillomaviruses marked with asterisk have yet to be classified by ICTV.

To investigate the evolutionary history of the seven novel Weddell seal papillomaviruses we constructed a phylogenetic tree based on the conserved E1, E2, and L1 ORFs (Gottschling et al., 2007) (Figure 4). With regards to the seven novel Weddell seal papillomaviruses, this tree is congruent with the L1 nucleotide tree (Figure 3). LwPV1 clusters with lambdapapillomaviruses (Figures 3 & 4) with the highest L1 amino acid pairwise identity (63.9%) with the L1 sequence of AmPV4 (Figure 2). Phylogenetically, LwPV3 is most closely related to ZcPV1, despite sharing the highest L1 amino acid pairwise identity with AmPV3 (63.3%). LwPV2 shares the highest amino acid pairwise identity (50%) with human papillomavirus (HPV) 4 in the *Gammapapillomavirus* genus, but phylogenetically it is most closely related to VvPV1, the sole member of *Treisetapapillomavirus genus* (Figures 3 & 4). The E7 protein of LwPV3 shares 50% pairwise identity with that of LwPV4 but in general is most closely related to ZcPV1 (Figures 2 - 4). The L1 protein of LwPV4 shared 63.4% with that of Felis catus papillomavirus (FcaPV2). LwPV5 clusters with taupapillomaviruses, sharing 57.9%, 57.6% and 56.2% amino acid pairwise identity with the L1, E1 and E2 sequences of canine papillomavirus (CPV) 17, CPV2, and CPV7, respectively. LwPV6-7 are most closely related to AmPV1 in the *Omegapapillomavirus* genus with an L1 amino acid pairwise identity of 68-69%.

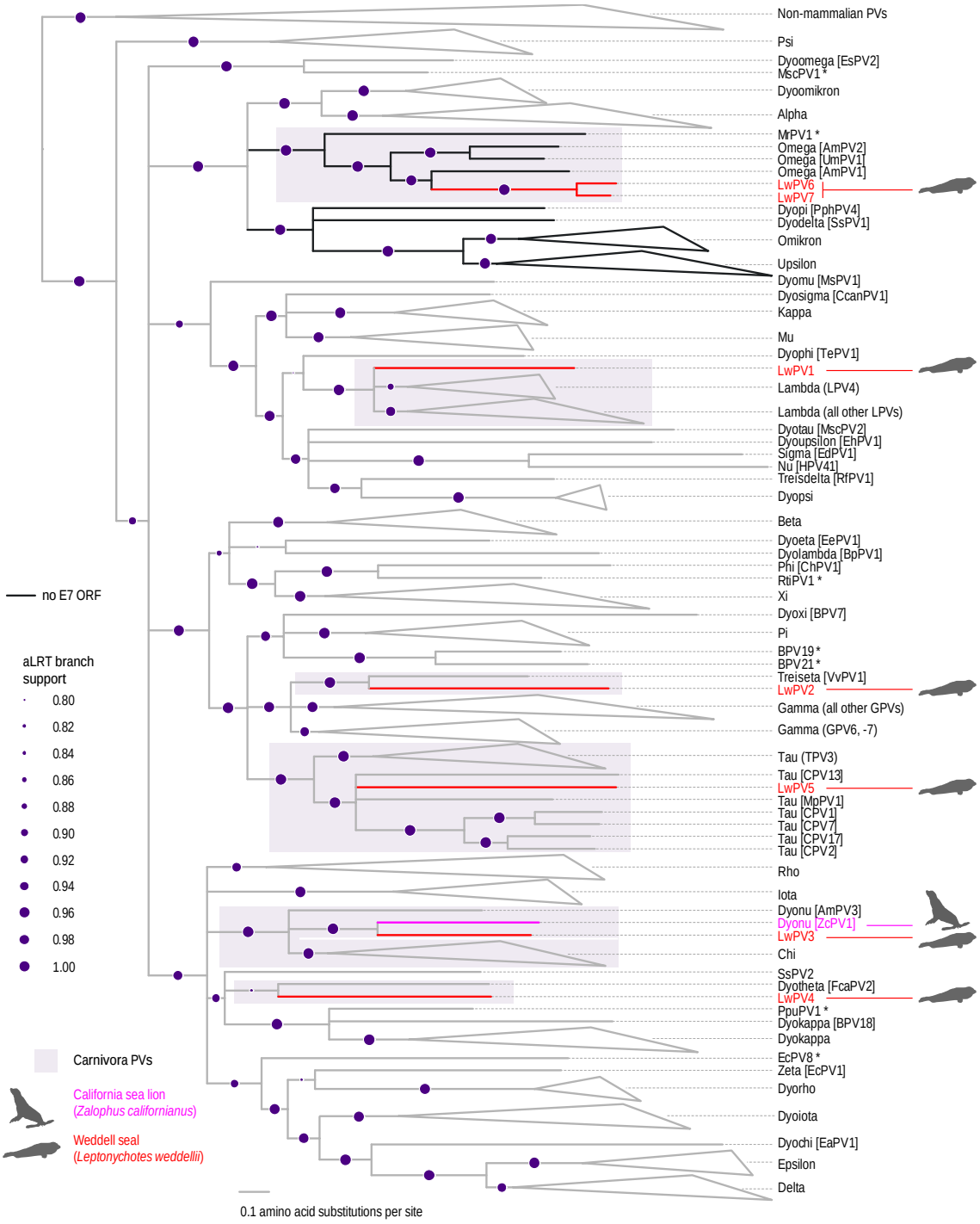

Figure 4: Maximum likelihood phylogenetic tree inferred using concatenated protein sequence of E1, E2 and L1. LwPV1-7 are highlighted in red and California sea lion papillomavirus 1 (ZcPV1) is highlighted in pink. Branches in black indicated lineages that have no recognisable E7 ORF. Branches with < 0.75 aLRT branch support have been collapsed. Branch support values indicated in purple circle size gradient. Papillomaviruses marked with asterisk are unclassified

3.2 Papillomaviruses lacking E7 gene

The oncoprotein E7 is not encoded by all papillomaviruses and studies based on the high risk HPVs have shown that this oncoprotein associates with cellular pRB through the conserved LxCxE motif (Roman and Munger, 2013). All classified members of the *Omega*- (including LwPV6-7), *Omikron*- and *Upsilonpapillomavirus* genera as well as Myotis ricketti papillomavirus (MrPV1) and Sus scrofa papillomavirus (SsPV1) lack the E7 ORF (Stevens et al., 2008a; Stevens et al., 2008b; Van Doorslaer, 2013; Wu et al., 2012; Zhang et al., 2017). The E7 ORF is also absent from all characterised cetacean papillomaviruses (Gottschling et al., 2011; Rector et al., 2008; Rehtanz et al., 2006; Robles-Sikisaka et al., 2012).

Bold branches in the phylogenetic trees presented in Figures 3 and 4 show lineages lacking an E7 gene. As depicted in the partition tree, the bolded clades have diverged from a recent common ancestor shared with alpha- and dyoomikronpapillomaviruses. Multiple gene loss events have been reported in the evolution of certain papillomavirus clades. Loss of the E6 gene has occurred on at least two occasions in the evolution of papillomaviruses. Gamma-6 papillomavirus species all lack E6 while it is present in all other gammapapillomaviruses, indicating a loss of E6 in the shared ancestor of *Gammapapillomaviruses* 6 (Van Doorslaer and McBride, 2016). Furthermore, loss of E6 has been hypothesized to have occurred twice in the evolution of *Xipapillomavirus* genera: once in the divergence of bovine papillomavirus 12 (BPV12) and again in the *Xipapillomvirus* 1 species (Van Doorslaer and McBride, 2016).

3.3 Papillomaviruses associated with the order Carnivora

Papillomaviruses in general appear to have co-evolved with their hosts, however, recombination, adaptive radiation, host switching and positive selection have been shown to contribute to their evolution of papillomaviruses (Burk et al., 2013; García-Vallvé et

al., 2005; Gottschling et al., 2007; Varsani et al., 2006). Pinnipeds belong to the order Carnivora, and their most recent common ancestor with Caniforms is approximately 45 mya (Higdon et al., 2007; McKenna and Bell, 1997). Among the Pinnipedia, the Antarctic Lobodontines originated and speciated in the late Miocene to early Pliocene, when the relative isolation around Antarctica allowed for rapid diversification of those individuals that could tolerate the relatively cold climate conditions that existed south of the Antarctic Circumpolar current (Higdon et al., 2007). To date, 40 papillomaviruses have been identified across 14 distinct carnivore hosts (Figures 5 &6). (Mengual-Chuliá et al., 2015; Ng et al., 2011; Rector and Van Ranst, 2013; Smits et al., 2013; Zhang et al., 2017). With the exception of UmPV1 (Polar bear; Omega), VvPV1 (red fox; Treiseta), FcaPV2 (Domestic cat; Dyotheta), and ZcPV1 (California Sea lion; Dyonu), all Carnivora associated papillomaviruses belong to the genera *Chi-*, *Lambda-* or *Taupapillomavirus*. ZcPV1, the only pinniped associated papillomavirus that was known until this study, is most closely related to chipapillomaviruses and belongs to the genus *Dyonupapillomavirus* (Rivera et al., 2012). All LwPVs cluster phylogenetically in clades containing papillomaviruses of closely related carnivore hosts (Figures 5 & 6). This is reminiscent of primate and artiodactyl papillomaviruses. Primate papillomaviruses form five defined clades, and within each genus, the viruses recapitulate the host evolutionary history (Van Doorslaer, 2013). It has previously been proposed that, similar to polyomaviruses (Buck et al., 2016), extant papillomavirus diversity can be explained by intra-host duplication followed by episodes of co-evolution (Bravo and Alonso, 2007; Van Doorslaer, 2013).

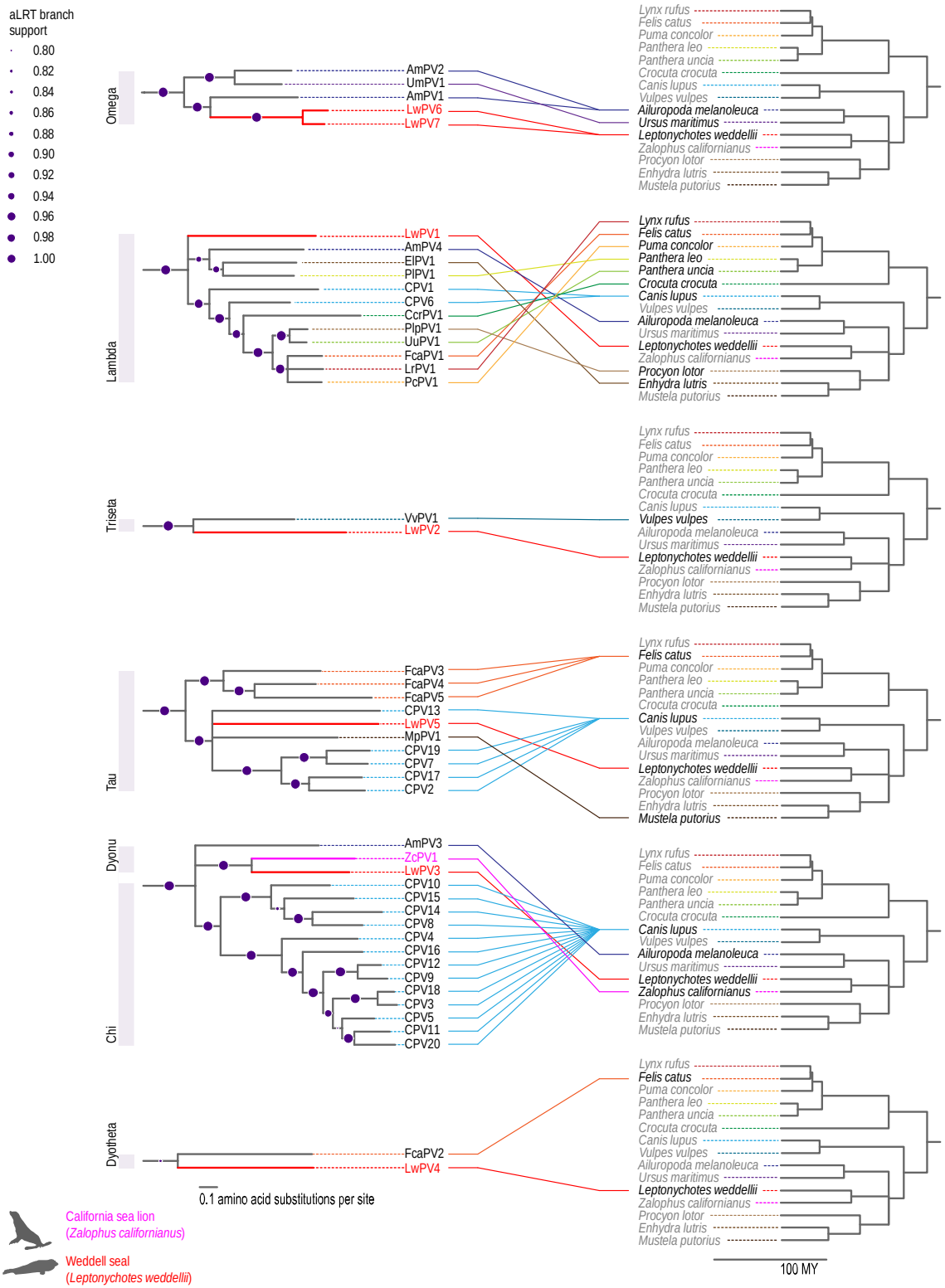

Figure 5: Co-evolution of carnivore papillomaviruses from Omega-, Lambda-, Triesta-, Tau-, Dyonu-, Chi- and Dyothetapapillomaviruses. Clades of concatenated E1-E2-L1 maximum likelihood phylogenetic tree (see figure 3). All Carnivora papillomaviruses known to date are present in the six clades and these are linked to their host phylogenies.

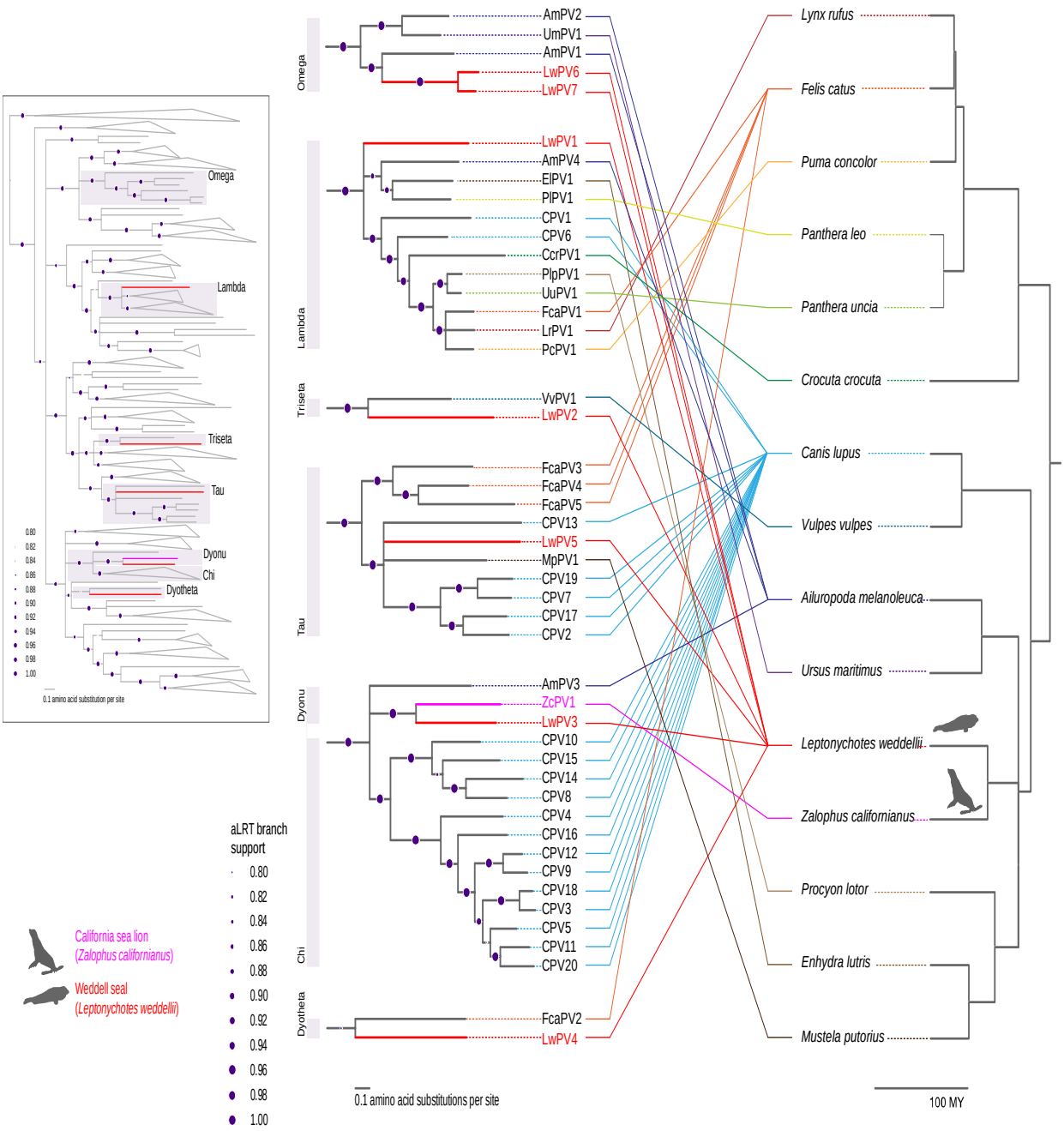

Figure 6: Concatenated E1-E2-L1 tree (Figure 3) in left hand box. Shaded clades where LwPVs cluster are shown in full with phylogenetic relationship to papillomavirus members links drawn to respective hosts in carnivore phylogenetic tree.

4. Concluding remarks

The papillomaviruses diversity in Weddell seals supports the presence of four or five distinct clades, corresponding to four or five ancestral viruses. We hypothesize that the first terrestrial animals were infected with at least four distinct papillomaviruses. As papillomaviruses co-evolved with these hosts, viral niche adaptation allowed for intra-host duplication (Van Doorslaer et al., 2015; Warren et al., 2015), in turn resulting in radiation and further host-parasite coevolution.

It has become evident that papillomaviruses identified in a single host may originate from multiple evolutionary lineages. This is shown for human papillomaviruses which are classified into five highly divergent genera, with the majority classified as *Alpha-*, *Beta-*, and *Gammapapillomavirus*. Similarly, canine papillomaviruses reveal at least three evolutionary lineages that are classified into distinct genera. This work has revealed that, even in a fairly small sample set, Weddell seals are similarly infected with a diverse set of papillomaviruses that are distinct from those found in other mammals. It is highly likely that these seven papillomaviruses are benign as no anogenital or oral cancers associate with Weddell seals were identified in this study nor have they been previously reported.

Metagenomics and high-throughput sequencing has exponentially increased the knowledge of Antarctic animal virology over the last five years (Smeele et al., 2018). Determining the viral diversity in this extreme and isolated habitat is important for monitoring animal health. Furthermore, expanding our understanding of carnivore papillomaviruses in a novel host has offered strong support for a gene loss event in the evolutionary history of papillomaviruses, thus extending our knowledge of the *Papillomaviridae* family.

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Data availability: All sequence data reported in this study has been deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under accession #s MG571089 - MG571095.

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Concluding chapter: Contributions and future directions

Antarctica has been an important environment for viral discovery in the last decade, despite the extremely harsh climate and limited wildlife. The research described here used metagenomics and high throughput sequencing to extend our knowledge of viruses among Antarctic animals in the Ross Sea, focusing on Weddell seals. Over three summer field seasons from 2014-2016, vaginal and nasal swabs from 81 Weddell seals were collected by Jennifer Burns' group. Previously, studies of Antarctic animal associated viruses have focused on avian viruses with little investigation to those associated with seals (Goraichuk et al., 2017; Hurt et al., 2016; Hurt et al., 2014; Miller et al., 2010; Neira et al., 2017; Thomazelli et al., 2010). Within the Ross Sea area, polyomaviruses have been identified in Adelie penguins, a Weddell seal and a sharp spinned notothen (Buck et al., 2016; Varsani et al., 2017; Varsani et al., 2015). Furthermore, two papillomaviruses have been characterized in Adelie penguins and various anelloviruses representing two species have been identified in Weddell seals (Fahsbender et al., 2017; Van Doorslaer et al., 2017; Varsani et al., 2014). These are the only viruses confirmed in Antarctic pinnipeds to date. The Ross Sea and McMurdo Sound represents a close-knit environment of marine, avian and seal life that interact. As Weddell seals are high trophic level predators in this ecosystem and remain in here year around (Testa and Siniff, 1987) they provide important insight to understanding this virome and monitoring health of the Ross Sea and McMurdo Sound.

Over the years, viral outbreaks have had devastating impacts on pinniped populations as well as captive individuals demonstrated by mass mortality events (Anthony et al., 2012; Butina et al., 2010; Inoshima et al., 2013; Kennedy et al., 2000; Laws and Taylor, 1957; Mamaev et al., 1996). Awareness of viruses among seal populations is therefore extremely important for monitoring health. An overview of all pinniped viruses identified to date is provided in Table 1. The viruses in bold representing those with fully sequenced genomes. In Antarctic seals, viruses are severely understudied with the majority of

information based on detection using serological approaches. The contribution this research has made to our knowledge of viral diversity in Antarctica is highlighted in red (Table 1). The recovery of seven novel papillomaviruses from Weddell seals inhabiting the Ross Sea thus extends our understanding of animal virology in the Antarctic as well as knowledge of papillomaviruses among pinnipeds. This is only the second study to identify papillomaviruses in seals revealing the otherwise lack in knowledge of these viruses in pinnipeds. Previously, only a single species of papillomavirus (ZcPV1) was found in two male California sea lions (Rivera et al., 2012). Although the papillomaviruses identified in this study cluster for the most part with established genera, it remains that at a sequence level WsPV1-7 are very distinct from known papillomaviruses and only distantly related to the papillomaviruses in their respective genera. This distant relationship is likely due to the long term geographical isolation of this Weddell seal population.

Table 1. Summary of all viruses associated with either free ranging or captive pinnipeds, those in bold resulting in whole genome sequencing and red revealing the viruses identified in this thesis

Pinniped species	Associated virus	Viral family	Viral detection	Sample and population	Reference
California sea lion (*Zalophus californianus*)	California sea lion polyomavirus (CSLPyV)	*Polyomaviridae*	Fibropapilloma mass, Electron microscopy observation of virions, DNA extraction RCA and partial sequencing	Tongue, small intestine and kidney samples from wild stranded individuals in California	(Colegrove et al., 2010; Wellehan et al., 2011)
	Zalophus californianus papillomavirus (ZcPV1)	*Papillomaviridae*	Histopathology, Whole genome sequencing	Swabs from perineal, axillary and prepuce lesions from two individuals in open ocean enclosure	(Rivera et al., 2012)
	Otarine adenovirus (OtAdV-1)	*Adenoviridae*	Genome sequencing, liver test, histopathology	Serum and tissue samples collected from Aquarium, stranded	(Goldstein et al., 2011; Inoshima et al., 2013)
	Zalophus californianus anellovirus (ZcAV)	*Anelloviridae*	ELISA, whole genome sequencing	Serum, Lung, tonsil, lymph-node, liver samples collected from stranded, zoo captive individuals	(Fahsbender et al., 2015; Ng et al., 2009)
	Otarine herpesvirus 1 (OtHV1)	*Herpesviridae*	PCR amplification of OtHV-1 terminase and DNA polymerase genes, Partial genome sequencing of DNA polymerase and terminase genes	Tissue samples from tumour masses in seals admitted to The Marine Mammal Center from along the central California Coastline	(King et al., 2002)
	Sea lion parapoxvirus (SLPV-1)	*Poxviridae*	Histopathology, Virion morphology with electron microscopy, partial sequencing	Skin nodule from head, neck and thorax collected from stranded on California coastline	(Nollens et al., 2006)
Steller sea lion (*Eumetopias jubatus*)	Poxvirus	*Poxviridae*	PCR targeting DNA polymerase gene and topoisomerase I gene	skin lesion samples collected from free-ranging and captive individuals	(Bracht et al., 2006)
	Parapoxvirus	*Poxviridae*	PCR targeting DNA polymerase gene and topoisomerase I gene	skin lesion samples collected from free-ranging and captive individuals	(Bracht et al., 2006)
	SSL Vesivirus	*Caliciviridae*	Viral isolation and electron microscopy, RT-PCR amplification of	Oral and rectal swabs and vesicular fluids from Steller sea lions in Alaska	(McClenahan et al., 2008)
Spotted seals (*Phoca largha*)	Parapoxvirus	*Poxviridae*	PCR targeting DNA polymerase gene and topoisomerase I gene	skin lesion samples collected from free-ranging and captive individuals	(Bracht et al., 2006)
Pacific harbor seal (*Phoca vitulina richardsii*)	Anellovirus (SealAV)	*Anelloviridae*	Metagenomics, whole genome sequencing	Lung samples collected during mortality of free-range population	(Ng et al., 2011)
	Harbor seal coronavirus (HSCoV)	*Coronaviridae*	Whole genome sequencing	Lung samples collected during mortality of free-range population	(Nollens et al., 2010)
Harbor seals (*Phoca vitulina*)	Influenza A virus (H3N8)	*Orthomyxoviridae*	Genome sequencing, hemagglutination assay, intravenous pathogenicity test, molecular pathology	Lung, lymph nodes, tonsil and kidney samples collected during mortality of free-range (Massachusetts)	(Anthony et al., 2012)

	Parapoxvirus	*Poxviridae*	Histopathology, virion morphology by electron microscopy, in situ hybridization,	Epithelial cells of stratum granulosum collected from individuals in rehabilitation center (Germany)	(Muller et al., 2003)
	Phocine herpesvirus 1 (PhHV-1)	*Herpesviridae*	Indirect enzyme-linked immunosorbent assay; real-time PCR assay and ELISA	Serum samples from wild and captive Canadian population; wild seals from Svalbard Norway	(Bellehumeur et al., 2016; Roth et al., 2013)
	Parvovirus	*Parvoviridae*	Next-generation sequencing (NGS), in situ hybridization	Lung and cerebral parenchyma samples from free-range population (Netherlands)	(Bodewes et al., 2013)
	Anellovirus	*Anelloviridae*	NGS, in situ hybridization	Lung samples from free-range population (Netherlands)	(Bodewes et al., 2013)
Hawaiian monk seal (*Neomonachus schauinslandi*)	Polyomavirus	*Polyomaviridae*	Histopathology, electron microscopy, partial genome sequencing	Liver, kidney and lung samples from captive (aquarium) seal	(Cortes-Hinojosa et al., 2016)
	Adenovirus	*Adenoviridae*	histopathology, electron microscopy, partial genome sequencing	Liver, kidney and lung samples from captive (aquarium) seal	(Cortes-Hinojosa et al., 2016)
South American fur seals (*Arctocephalus australis*)	Adenovirus	*Adenoviridae*	Partial genome sequencing, qPCR assay, histopathology,	Nasal fecal and liver samples from Free-range (Peruvian pup population)	(Hinojosa, 2014)
	sequences of Anelloviridae and parvoviridae families detected in metagenomic study	*Anelloviridae*	Whole genome sequencing with Ion Torrent and Illumina platforms	Fecal samples from deceased individuals along shore	(Kluge et al., 2016)
Northern elephant seals (*Mirounga angustirostris*)	H1N1 influenza virus	*Orthomyxoviridae*	Serology, whole genome sequencing	Nasal swabs and serum from Free-ranging individuals	(Goldstein et al., 2013)
Subantarctic fur seal (*Arctocephalus tropicalis*)	Sequences of Anelloviridae and parvoviridae families detected in metagenomic study	*Anelloviridae*	Whole genome sequencing with Ion Torrent and Illumina platforms	Fecal samples from deceased individuals along shore	(Kluge et al., 2016)
Hooded seals (*Cystophora cristata*)	Phocid herpesvirus 1 (PhoHV-1)	*Herpesviridae*	Indirect enzyme-linked immunosorbent assay, partial sequencing of herpes DNA polymerase	Serum samples collected from Canadian population	(Bellehumeur et al., 2016)
Harp seal (*Pagophilus groenlandica*)	Phocid herpesvirus 1 (PhoHV-1)	*Herpesviridae*	Indirect enzyme-linked immunosorbent assay, partial sequencing of herpes DNA polymerase	Serum samples collected from Canadian population	(Bellehumeur et al., 2016)
Grey seals (*Halichoerus grypus*)	Phocid herpesvirus 1 (PhoHV-1)	*Herpesviridae*	Indirect enzyme-linked immunosorbent assay	Serum samples collected from Canadian population	(Bellehumeur et al., 2016)
	Parapoxvirus	*Poxviridae*	Electron microscopy	Outbreak in population off the coast of Cornwall	(Simpson et al., 1994)
Ringed seal (*Phoca hispida*)	Phocid herpesvirus 1 (PhoHV-1)	*Herpesviridae*	Indirect enzyme-linked immunosorbent assay,	Serum samples from wild population; Ulukhaktok, Northwest Territories, Canada	(Bellehumeur et al., 2016)

			partial sequencing of herpes DNA polymerase		
	Seal picornavirus 1 (SePV-1)	*Picornaviridae*	Random amplification and sequencing, RT-PCR of SePV-1 region and sequencing.	Nasal swab from a seal hunted in 2002 around Ulukhaktok, Beaufort Sea	(Kapoor et al., 2008)
South American sea lion (*Otaria flavescens*)	Otarine adenovirus	*Adenoviridae*	Histopathology, liver function tests, genome sequencing	Serum and tissue of seal with hepatitis in Japanese aquarium	(Inoshima et al., 2013)
South African fur seal (*Arctocephalus pusillus*)	Otarine adenovirus	*Adenoviridae*	Histopathology, liver function tests, genome sequencing	Serum and tissue of seal with hepatitis in Japanese aquarium	(Inoshima et al., 2013)
Northern fur seal (*Callorhinus ursinus*)	Polyomavirus	*Polyomaviridae*	Transmission electron microscopy, genome sequencing of VP1	Placenta of a single newborn pup around Pribilof Islands, Alaska	(Duncan et al., 2013)
	Otarine herpesvirus 4 (OtHV4)	*Herpesviridae*	Partial sequencing of polymerase gene and glycoprotein B	Vaginal swabs	(Cortes-Hinojosa et al., 2016)
Weddell seal (*Leptonychotes weddellii*)	Parapoxvirus	*Poxviridae*	Electron microscopy, partial sequencing	Neck skin lesion from deceased individual Queen Maud Land, Antarctica	(Tryland et al., 2005)
	Phocid alphaherpesvirus 1	*Herpesviridae*	Histopathology, Partial genome sequencing	Skin lesions of seals around Queen Maud Land, Antarctica	(Harder et al., 1991; Stenvers et al., 1992; Tryland et al., 2012)
	Phocine distemper virus (PDV)	*Paramyxoviridae*	Serology	Serum taken from seals around East Antarctica	(McFarlane, 2009)
	Torque teno Leptonychotes weddelli virus 1, -2 (TTLwV1, TTLwV2)	*Anelloviridae*	HTS-informed approach, genomes recovered using abutting primers, cloned and Sanger sequenced.	Vaginal, nasal, and faecal samples collected around the Ross Sea during summer field season of 2014	(Fahsbender et al., 2017)
	Polyomavirus (WsPyV)	*Polyomaviridae*	Metagenomics and high-throughput sequencing	Kidney sample of deceased seal around Ross Sea, Antarctica	(Varsani et al., 2017)
	Leptonychotes weddelli papillomavirus 1-7 (LwPV1-7)	*Papillomaviridae*	Metagenomics and high-throughput sequencing	Vaginal and nasal swabs from seals around Ross Sea, Antarctica collected during summer field season between 2014-2016	
Leopard seal (*Hydrurda leptonyx*)	Canine distemper virus (CDV)	*Paramyxoviridae*	Microneutralization test used to detect CDV-like antibodies with two CDV strains and PDV isolate	Serum samples collected around Antarctic peninsula in 1989	(Bengtson et al., 1991)
Crabeater seal (*Lobodon carcinophagus*)	Phocid alphaherpesvirus 1	*Herpesviridae*	Histopathology, Partial genome sequencing	Skin lesions of seals around Queen Maud Land, Antarctica	(Harder et al., 1991; Tryland et al., 2012)
	Canine distemper virus (CDV)	*Paramyxoviridae*	Microneutralization test used to detect CDV-like antibodies with two CDV strains and PDV isolate	Serum samples collected around Antarctic peninsula during January/ March 1989	(Bengtson et al., 1991)
Ross seal (*Ommatophoca rossii*)	Phocid alphaherpesvirus 1	*Herpesviridae*	Histopathology, Partial genome sequencing	Skin lesions of seals around Queen Maud Land, Antarctica	(Tryland et al., 2012)

Caspian seal (*Phoca caspica*)	Canine distemper virus (CDV)	*Paramyxoviridae*	Serologic examination, RT-PCR, sequencing P gene fragment	Serum and tissue samples collected during mass mortality event around Caspian Sea in 1997	(Kennedy et al., 2000)
Atlantic Walrus (*Obodenus rosmarus rosmarus*)	Phocine distemper virus (PDV)	*Paramyxoviridae*	Virus neutralization test for CDV, measles virus, peste des petits ruminants virus, phocine distemper virus, and rinderpest virus	Serum samples of three male Atlantic walruses sampled in Hudson Strait, south of Nottingham Island, Canada t	(Duignan et al., 1994)
Walrus (*Obodenus rosmarus*)	Walrus calicivirus (WCV)	*Caliciviridae*	Viral isolation, Suppression Subtractive Hybridization (SSH), RT-PCR on extracted RNA, amplified by SMART PCR cDNA synthesis kit	Walrus feces from resting sea ice In south central Chukchi Sea, 1977	(Ganova-Raeva et al., 2004)
Antarctic fur seal (Arctocephalus gazelle)	Phocid alphaherpesvirus 1	*Herpesviridae*	iELISA using PhHV-1 antigen	Serum samples collected around Bouvet, sub- Antarctic 2000-2001, 2001-2002	(Tryland et al., 2012)
Siberian seal (*Phoca sibirica*)	Canine distemper virus (CDV)	*Paramyxoviridae*	RT-PCR on samples specific for DMV, PMV, and PDV. Amplicons were for a fragment of the phosphoprotein gene. Analyzed by direct sequencing on a Beckman Coulter 8800 automated sequencer	Forty-two samples of brain and spleen collected during 2000-2007	(Butina et al., 2010)
South American Fur seals (*Arctocephalus australis*)	Adenovirus	*Adenoviridae*	Nested-PCR for amplification of DNA polymerase gene using primers for all members of *Mastadenovirus* genus. Amplicons cloned and sequenced	21 fecal samples collected from South American fur seals deceased along the coast of Rio Grande do Sul State, Southern Brazil.	(Chiappetta et al., 2017)
	Fur seal feces-associated circoviridae	*Circoviridae*	Nested-PCR using degenerate primers to amplify a segment of the rep gene conserved in Circoviruses and Cycloviruses. Amplicons cloned and sequenced	21 fecal samples collected from South American fur seals deceased along the coast of Rio Grande do Sul State, Southern Brazil.	(Chiappetta et al., 2017)
Southern elephant seal (*Mirounga leonina*)	Southern elephant seal virus (SES virus)	*Togaviridae*	Virus from blood sucking lice cultured in BHK-21 cells. Used in viral neutralization assay for serology and negative stain electron microscopy. RT-PCR and Sanger sequencing of capsid protein.	Blood sucking lice (*Lepidophthirus macrorhini*) and serum samples collected around Macquarie Island	(Forrester et al., 2011; La Linn et al., 2001)
New Zealand fur seal (*Arctocephalus forsteri*)	Fur seal feces-associated circular DNA virus	Unclassified	Metagenomics and High-throughput sequencing	A fecal sample collected from a New Zealand fur seal off the coast of Kaikoura, NZ in October 2012	(Sikorski et al., 2013)

This study demonstrates that papillomaviruses have a far greater diversity among seals than a single lineage. The six distinct lineages observed for LwPV1-6 and 7 indicates that there were at least six variants of these papillomaviruses in the last common ancestor of pinnipeds. These lineages span widely over the phylogenetic trees with LwPV1- 7 phylogenetically clustered into clades consisting of closely related carnivore host papillomaviruses. Given the co-evolution of papillomaviruses with their hosts, this association was expected. However, within these clades LwPV1- 7 reveal a distant divergence from other members perhaps due to the geographical isolation of Weddell seals. Different lineages of papillomaviruses within the same host is observed frequently among this family of viruses and has been shown for HPVs that reveal three major lineages of papillomaviruses. The identification of LwPV1-7 now draws attention to six lineages of papillomaviruses that have infected an ancient common ancestor to carnivores and diversified in these hosts. The intrigue lies in that these six highly diverse lineages of Weddell seal papillomaviruses was identified within a limited number of sampled individuals from relatively a small population.

This discovery of highly diverse papillomaviruses among Weddell seals has revealed trends in the broader evolutionary patterns of papillomaviruses. Gene loss in papillomaviruses is reported among certain clades typically involving E6 or E7 (Gottschling et al., 2011; Rector et al., 2008; Rehtanz et al., 2006; Robles-Sikisaka et al., 2012; Stevens et al., 2008a; Stevens et al., 2008b; Van Doorslaer and McBride, 2016; Wu et al., 2012; Zhang et al., 2017). These encode oncoproteins that bind p53 and pRB, respectively, key proteins involved in cell cycle regulation. The influence of this gene loss on infection or pathogenesis is not understood. The lack of the E7 gene within LwPV6 and 7 and other members of *Omegapapillomavirus* supports the loss of the E7 gene from this genus that until now was only known to include bear papillomaviruses. Phylogenetic analysis of the E1 E2 L1 concatenated sequences revealed that other clades missing this E7 gene such as Dyopi-, Dyodelta, Omicron- and Upsilonpapillomavirus share a recent common ancestor with Omega-, Alpha- and Dyoomikronpapillomavirus. However it is likely the most recent common ancestor of Alpha and Dyoomikronpapillomavirus encoded the E7 gene given papillomaviruses in these genera encode an E7 gene and it is in the genera Omega-, Dyopi-, Dyodelta-, Omicron- and Upsilonpapillomavirus E7 has been lost. While the discovery of LwPV6 and 7 provide

further phylogenetic support for the loss of E7 from *Omegapapillomavirus* and the evolutionary relationship to other E7 absent genera, it raises questions of what drove this gene loss in these clades and how this affected pathogenicity of these papillomaviruses.

The phylogenetic relationship between LwPV2 and VvPV-1 and the significant pairwise identities shared with LwPV2 and gammapapillomavirus HPVs may support host switching of an HPV to an ancient ancestor of seals and canines. However, the phylogenetic analysis presented here shows a split in the gammapapillomavirus clade raising questions of the taxonomic classification of this genus.

During breeding season, females aggregate together into colonies allowing males to mate with multiple females (Stirling, 1969). This could potentially result in transmission of papillomaviruses between individuals through contact with infected mucosa. L1 specific primers were used to screen individuals for the presence of LwPV1-7 in order to gain an understanding of their prevalence among individuals. While the same individuals waere not sampled across the different field seasons each Weddell seal papillomavirus was detected in a single individual and only in samples from a single field season. This may indicate papillomavirus transmission is uncommon between female Weddell seals, however our dataset lacks information of papillomaviruses among males or offspring and thus potential transmission to these individuals in the population that may impact these papillomaviruses being maintained among certain individuals. Using this data to screen samples from males or offspring in this region could offer a greater understanding as to the prevalence of these papillomaviruses in the population and whether they may be sexually or congenitally transmitted. Furthermore, whether individuals in other populations of Weddell seals that inhabit the Vestfolds of Antarctica and the sub-Antarctic Signy Island also contain papillomaviruses is unknown. Such research would be interesting in order to demonstrate whether papillomaviruses are widely dispersed among all populations of Weddells seals and whether they follow similar evolutionary lineages. However, it is important to keep in mind that due to the harsh conditions and conservation regulations sampling of Antarctic animals is highly restrictive and therefore this dataset provides valuable information toward viruses circulating in this population of Weddell seals.

It is likely that this research has only scratched the surface of the diversity of papillomaviruses in seals considering the extremely high diversity within a relatively

small and isolated population which in turn contributes to the limited understanding of the Antarctic virome through confirmed sequence data. In terms of the evolution of *Papillomaviridae* the discovery of these seven genomes also supports that gene loss has been a driver for diversification of papillomaviruses in certain genera and that papillomaviruses likely have multiple evolutionary lineages within a single host. Both of these influences to our understanding of the mechanisms of papillomavirus evolution draw attention to issues in our current taxonomic classification of this viral family.

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