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Short Communication

Identification of an avian polyomavirus associated with Adélie penguins (*Pygoscelis adeliae*)

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Little is known about viruses associated with Antarctic animals, although they are probably widespread. We recovered a novel polyomavirus from Adélie penguin (*Pygoscelis adeliae*) faecal matter sampled in a subcolony at Cape Royds, Ross Island, Antarctica. The 4988 nt Adélie penguin polyomavirus (AdPyV) has a typical polyomavirus genome organization with three ORFs that encoded capsid proteins on the one strand and two non-structural protein-coding ORFs on the complementary strand. The genome of AdPyV shared ~60% pairwise identity with all avipolyomaviruses. Maximum-likelihood phylogenetic analysis of the large T-antigen (T-Ag) amino acid sequences showed that the T-Ag of AdPyV clustered with those of avipolyomaviruses, sharing between 48 and 52% identities. Only three viruses associated with Adélie penguins have been identified at a genomic level, avian influenza virus subtype H1N2 from the Antarctic Peninsula and, respectively, *Pygoscelis adeliae* papillomavirus and AdPyV from capes Crozier and Royds on Ross Island.

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There is extremely little information about pathogens and parasites associated with Antarctic animals (Barbosa & Palacios, 2009; Kerry & Riddle, 2009). Of all Antarctic vertebrates, the penguins are possibly the best studied. Antarctic penguins have been showing signs of disease of unknown pathology in recent years, in particular unexplained incursions of feather loss in Adélie penguins (*Pygoscelis adeliae*) and Emperor penguins (*Aptenodytes forsteri*) in the Ross Sea (Ainley/Ballard Antarctic field team observations at Cape Crozier for Adélie penguins for 2011/2012 season and personal communication with Gerald L. Kooyman for Emperor penguins observed in mid-1990s at Cape Washington) and also as reported by Barbosa *et al.*

(2014) in the Antarctic Peninsula. Similar feather-loss patterns have been noted previously in African penguins (*Spheniscus demersus*) at rehabilitation centres and in Magellanic penguins (*Spheniscus magellanicus*) in colonies in Argentina (Kane *et al.*, 2010).

Despite the wealth of information on penguin biology and ecology, there is limited information about their viruses. Most of the early studies have relied on serological assays for identifying putative paramyxoviruses, orthomyxoviruses, flaviviruses and birnaviruses in wild penguin populations (Alexander *et al.*, 1989; Austin & Webster, 1993; Gardner *et al.*, 1997; Miller *et al.*, 2010; Morgan & Westbury, 1981; 1988; Morgan *et al.*, 1981, 1985; Smith *et al.*, 2008; Thomazelli *et al.*, 2010) and herpesviruses and togaviruses in captive individuals (Kincaid *et al.*, 1988; Tuttle *et al.*, 2005). A handful of recent studies have identified some of the viruses (avipoxviruses, Newcastle disease

The GenBank/EMBL/DDBJ accession number for the genome sequence of the Adélie penguin polyomavirus is KP033140.

One supplementary figure and one supplementary table are available with the online Supplementary Material.

viruses, adenovirus, avian influenza virus and papillomavirus) at a molecular level (Carulei *et al.*, 2009; Hurt *et al.*, 2014; Kane *et al.*, 2012; Lee *et al.*, 2014; Thomazelli *et al.*, 2010; Varsani *et al.*, 2014).

Our team recently identified a novel papillomavirus associated with Adélie penguins breeding at Cape Crozier, Ross Island, Antarctica (Varsani *et al.*, 2014). Here, we report investigations at Cape Royds, a much smaller colony (~3000 vs 280 000 pairs) on the opposite coast of the island (Lyver *et al.*, 2014), where conditions are much more severe (Dugger *et al.*, 2014). As part of an ongoing diet study in a subcolony at Cape Royds, we had access to faecal matter from which we could identify prey hard parts as well as viral pathogens. We placed a 1 m² tray on the ground within an area where Adélie penguins nest. The tray, constructed of a hardwood frame holding a densely meshed (mesh ~0.5 mm), stainless steel hardware cloth, was placed before breeding commenced to passively collect faecal material over the 2012/2013 breeding season. Adélie penguins build their nests over the tray and defecate onto it through the course of the ~3 month breeding season. Furthermore, the penguins in their subcolonies nest at high density and thereby protect their nests from predators and competitors. Thus, faecal samples from the tray may originate from multiple penguin territories, but they are exclusively from Adélie penguins. We collected faeces from the tray at the end of the season (late January) once the nests were unoccupied.

At the end of the 2012/2013 Adélie penguin breeding season, ~50 ml of the faecal matter was transferred from the tray into 50 ml sterile tubes using sterile wooden spatulas. The faecal samples were stored and transferred to the laboratory in a frozen state. Approximately half of the faecal matter from the tray was resuspended and homogenized in 50 ml SM buffer [0.1 M NaCl, 50 mM Tris/HCl (pH 7.4), 10 mM MgSO₄] and processed as described previously (Varsani *et al.*, 2014). In brief, the supernatant of the homogenate was recovered after centrifugation at 10 000 g for 20 min. This was then sequentially filtered through 0.45 and 0.2 µm (pore size) syringe filters and 3 g of PEG 8000 (Sigma) was mixed gently with 20 ml filtrate. The 15% (w/v) PEG 8000 filtrate solution was then centrifuged for 20 min at 10 000 g and the pellet was resuspended in 2 ml SM buffer at 4 °C overnight. A High Pure Viral Nucleic Acid kit (Roche Diagnostics) was used to purify viral DNA from 200 µl of the final resuspended solution. We used rolling-circle amplification with a TempliPhi kit (GE Healthcare) to enrich for circular DNA, and this was sequenced on an Illumina HiSeq 2000 sequencer at the Beijing Genomics Institute (Hong Kong). ABySS v.1.3.5 (Simpson *et al.*, 2009) was used to *de novo* assemble (kmer=64) the paired-end reads. The translation products of the assembled contigs that were >500 nt were analysed for similarity to known viral proteins using BLASTX (Altschul *et al.*, 1990). Translation products of a 4981 nt *de novo*-assembled contig were found to have significant similarities to proteins encoded by avian polyomaviruses.

Polyomaviruses are non-enveloped viruses (~40–45 nm in diameter) with circular dsDNA genomes (~4600–5700 nt). Their genomes are bidirectionally transcribed and encode three structural proteins (VP1, VP2 and VP3) from one strand, which assemble to form the icosahedral viral capsid, and at least two non-structural proteins on the complementary strand (large and small tumour antigens: T-Ag and t-Ag, respectively). T-Ag and t-Ag are transcribed early in infection. In a few mammalian polyomaviruses and some avian polyomaviruses, an additional ORF is expressed (VP4) that is thought to play a role in capsid assembly and release (Gerits & Moens, 2012).

Polyomaviruses are known to infect a wide range of mammalian and avian hosts. Apart from four human polyomaviruses that belong to the genus *Wukipolyomavirus* (*WU polyomavirus*, *KI polyomavirus*, *Human polyomavirus 6* and *Human polyomavirus 7*), all remaining mammalian polyomaviruses belong to the genus *Orthopolyomavirus*. All the avian-infecting polyomaviruses belong to the genus *Avipolyomavirus* (Johns *et al.*, 2011). Six avipolyomavirus species have been identified to date, namely: *Avian polyomavirus* (infecting various parrot species) (Müller & Nitschke, 1986), *Goose hemorrhagic polyomavirus* (infecting geese and ducks) (Guerin *et al.*, 2000), *Finch polyomavirus* (infecting *Pyrrhula pyrrhula griseiventris*), *Crow polyomavirus* (infecting *Corvus monedula*) (Johns *et al.*, 2006), *Canary polyomavirus* (infecting *Serinus canaria*) (Halami *et al.*, 2010) and *Butcherbird polyomavirus* (infecting *Cracticus torquatus*) (Bennett & Gillett, 2014). Avian polyomaviruses are known to cause inflammatory disease in birds; in some species the acute clinical disease can result in high mortality (Guerin *et al.*, 2000; Johns & Müller, 2007; Krautwald *et al.*, 1989) and in some species chronic disease of skin and feathers (Krautwald *et al.*, 1989; Wittig *et al.*, 2007).

Based on the 4981 nt *de novo*-assembled contig sequence encoding a putative T-Ag, we designed a set of abutting primers Pry-AP-F: 5'-GCATCCAAGGCTGAGGTCCAA-GCCG-3' and Pry-AP-R: 5'-CCCGATGGGGATTTCAG-CAGC-3' to recover the complete viral genome using KAPA Hifi Hotstart DNA polymerase (Kapa Biosystems) and using the following protocol: initial denaturation at 95 °C for 3 min, followed by 25 cycles of 98 °C for 20 s, 60 °C for 15 s and 72 °C for 5 min and a final extension at 72 °C for 5 min. The resulting amplicon was resolved on a 0.7% agarose gel (stained with SYBR Safe DNA gel stain), excised, gel purified and cloned into the pJET1.2 plasmid (ThermoFisher). The recombinant plasmid containing the amplicon was Sanger-sequenced by primer walking at Macrogen Inc. (South Korea). The Sanger-sequenced contigs were assembled using DNABaser v.4 (Heracle BioSoft S.R.L.). All nucleotide and amino acid pairwise identities were calculated using SDT v.1.2 (Muhire *et al.*, 2014).

A simple analysis of the 4988 nt viral sequence revealed that its genome architecture was similar to polyomaviruses and that its genome shared ~60% pairwise identity with the genomes of other avipolyomaviruses (Fig. 1). We have

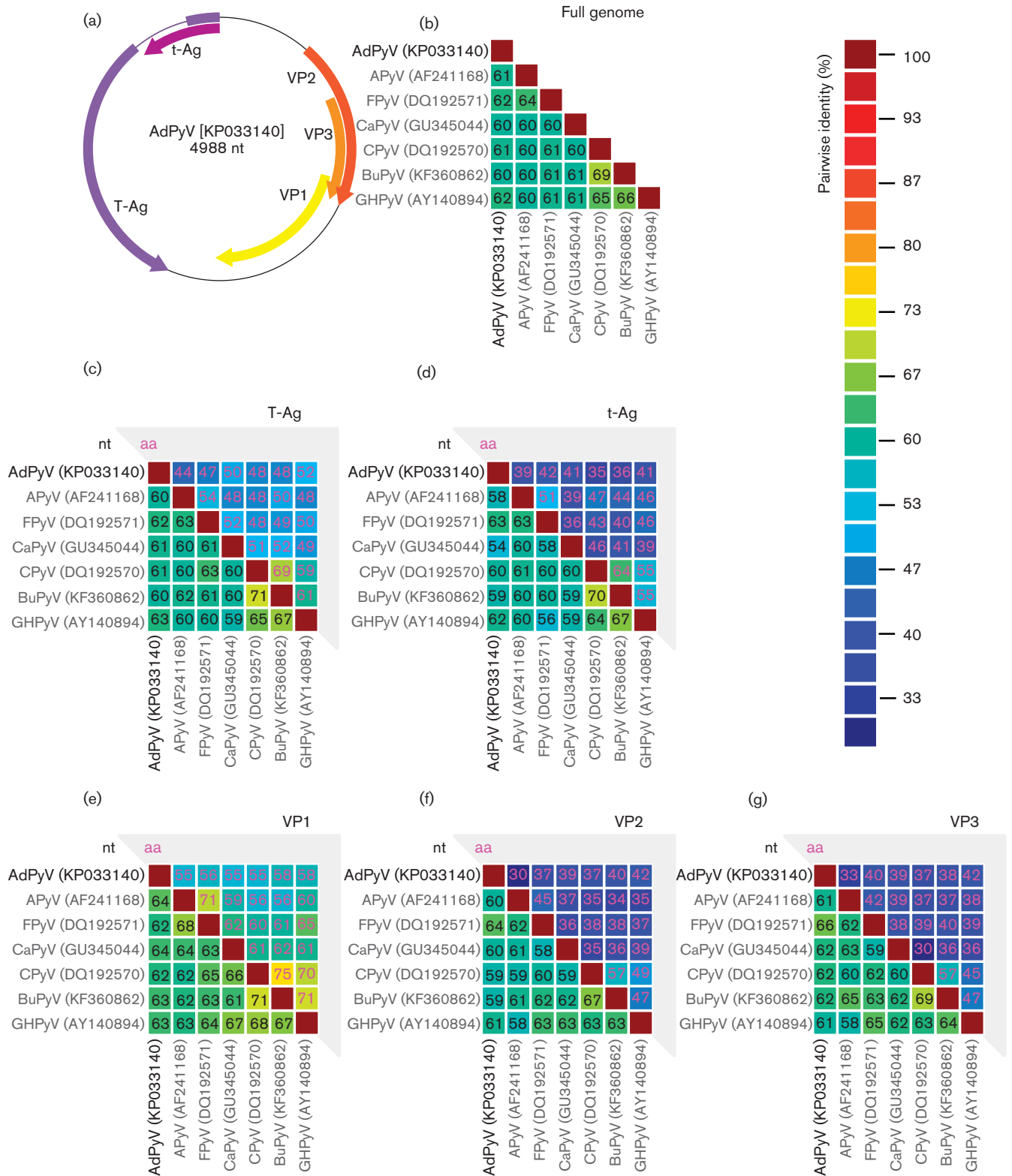


Fig. 1. (a) Genome organization of Adélie penguin polyomavirus (AdPyV). (b) Percentage pairwise identities of the full genomes of the avipolyomaviruses. (c–g) Nucleotide (nt) and amino acid (aa) percentage pairwise identities of the avipolyomavirus sequences of the large T-Antigen (T-Ag) (c), small T-Antigen (t-Ag) (d), VP1 (e), VP2 (f) and VP3 (g). APyV, *Avian polyomavirus*; FPyV, *Finch polyomavirus*; CaPyV, *Canary polyomavirus*; CPyV, *Crow polyomavirus*; BuPyV, *Butcherbird polyomavirus*; GHPyV, *Goose hemorrhagic polyomavirus*.

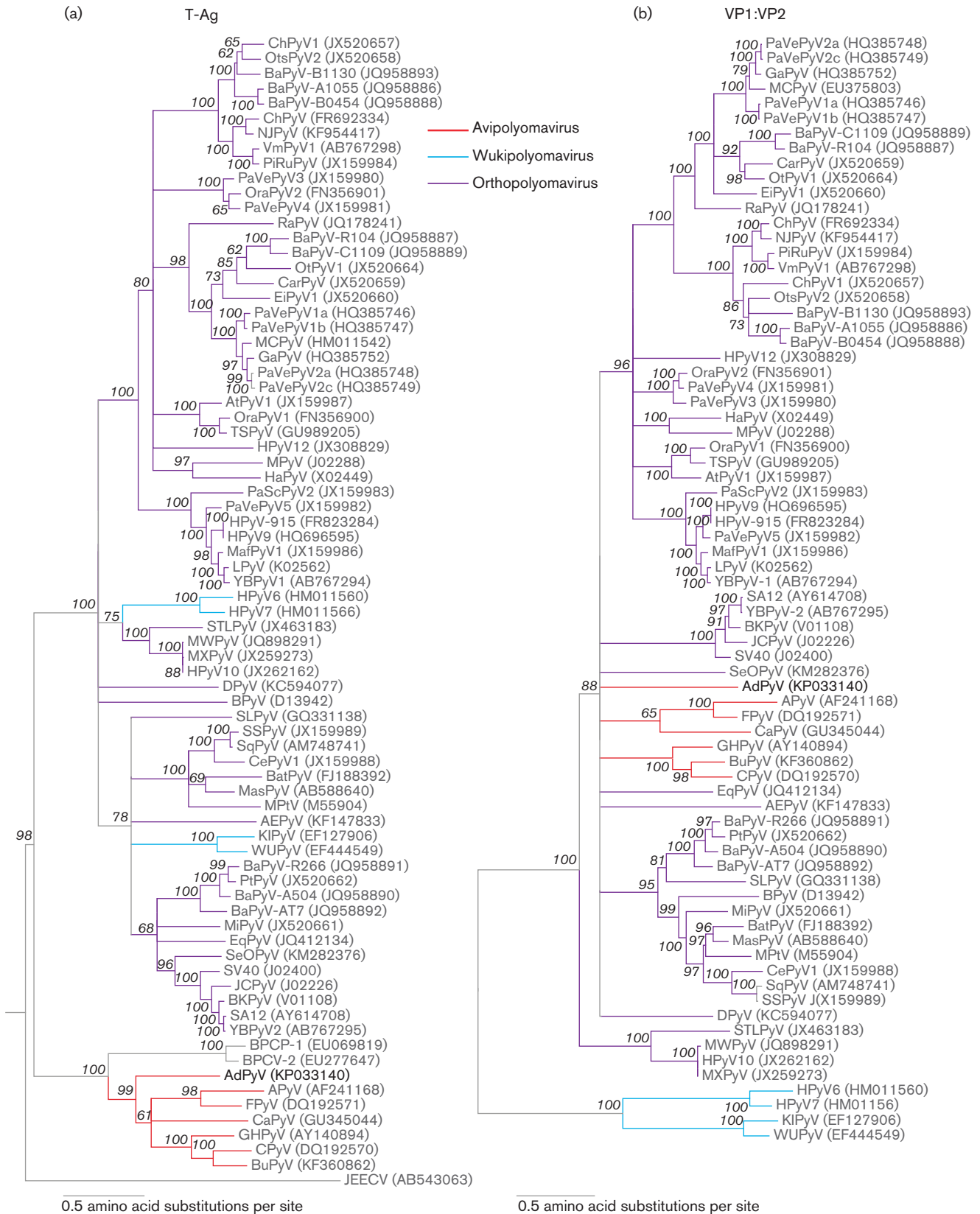


Fig. 2. ML phylogenetic trees of the large T-Antigen (T-Ag) (a) and VP1 : VP2 concatenated amino acid sequences (b). The T-Ag ML phylogenetic tree was rooted with papillomavirus E1 sequences and the VP1 : VP2 tree was mid-point rooted. Branches with <60% bootstrap support have been collapsed. See Table S1, available in the online Supplementary Material, for full names.

tentatively named this viral genome Adélie penguin polyomavirus (AdPyV). The T-Ag (1983 nt), t-Ag (537 nt), VP1 (1083 nt), VP2 (1050 nt) and VP3 (705 nt) share ~54–64 % pairwise nucleotide identity and ~30–58 % amino acid identity with homologous ORFs encoded by avipolyomaviruses (Fig. 1).

We also undertook posterior mapping of paired-end reads from Illumina sequencing of the 4988 nt AdPyV genome using Bowtie 2 v.2.2.3 (Langmead & Salzberg, 2012). The reads mapped across the whole genome with >250-fold coverage (Fig. S1). The *de novo*-assembled contig was 99.9 % similar to the PCR-recovered and cloned genome that was Sanger-sequenced.

Given that there is some movement of individual penguins between colonies on Ross Island (Dugger *et al.*, 2010), we screened the faecal sample from Cape Crozier reported by Varsani *et al.* (2014) and also checked the sequence reads resulting from this sample for AdPyV but found no evidence of it in the sample. Similarly, we did not find any evidence of the *P. adeliae* papillomavirus (Varsani *et al.*, 2014) in the Cape Royds sample.

We assembled two datasets of amino acid sequences, the T-Ags and concatenated VP1:VP2 of representative wuki-, ortho- and avipolyomaviruses. In the T-Ag dataset, we also

included the T-Ag-like sequences encoded by Bandicoot papillomatosis carcinomatosis virus 1 and 2 (BPCV-1 and -2, respectively) (Woolford *et al.*, 2007) and Japanese eel endothelial cells-infecting virus (Mizutani *et al.*, 2011) and selected avian papillomavirus E1 sequences (to root the tree). These datasets were aligned using MUSCLE (Edgar, 2004). The alignments were used to infer maximum-likelihood (ML) phylogenetic trees using PhyML 3.0 (Guindon *et al.*, 2010); 1000 bootstrap replicates using the RtRev+G+I+F model of substitution (for both aligned datasets) chose the best-fit model using ProTest (Darriba *et al.*, 2011). The T-Ag ML phylogenetic tree was rooted with the papillomavirus E1 sequences, whereas the VP1:VP2 one was mid-point rooted. All branches with <60 % bootstrap support were collapsed using Mesquite v.2.7 (<http://mesquiteproject.org/>). The ML phylogenetic analysis of the T-Ag clearly showed that the AdPyV clusters with other avipolyomaviruses (Fig. 2). As noted previously (Woolford *et al.*, 2007), the T-Ag-like protein sequence encoded by BPCV-1 was phylogenetically basal to the avipolyomaviruses (Fig. 2). Within the protein sequences of the T-Ag, we identified the polyomavirus conserved region 1 (CR1; LIRLL) and hexapeptide (HPDKGG), retinoblastoma protein binding motif (pRB; LYCEE), a putative nuclear localization signal (NLS; TPK--NKP),

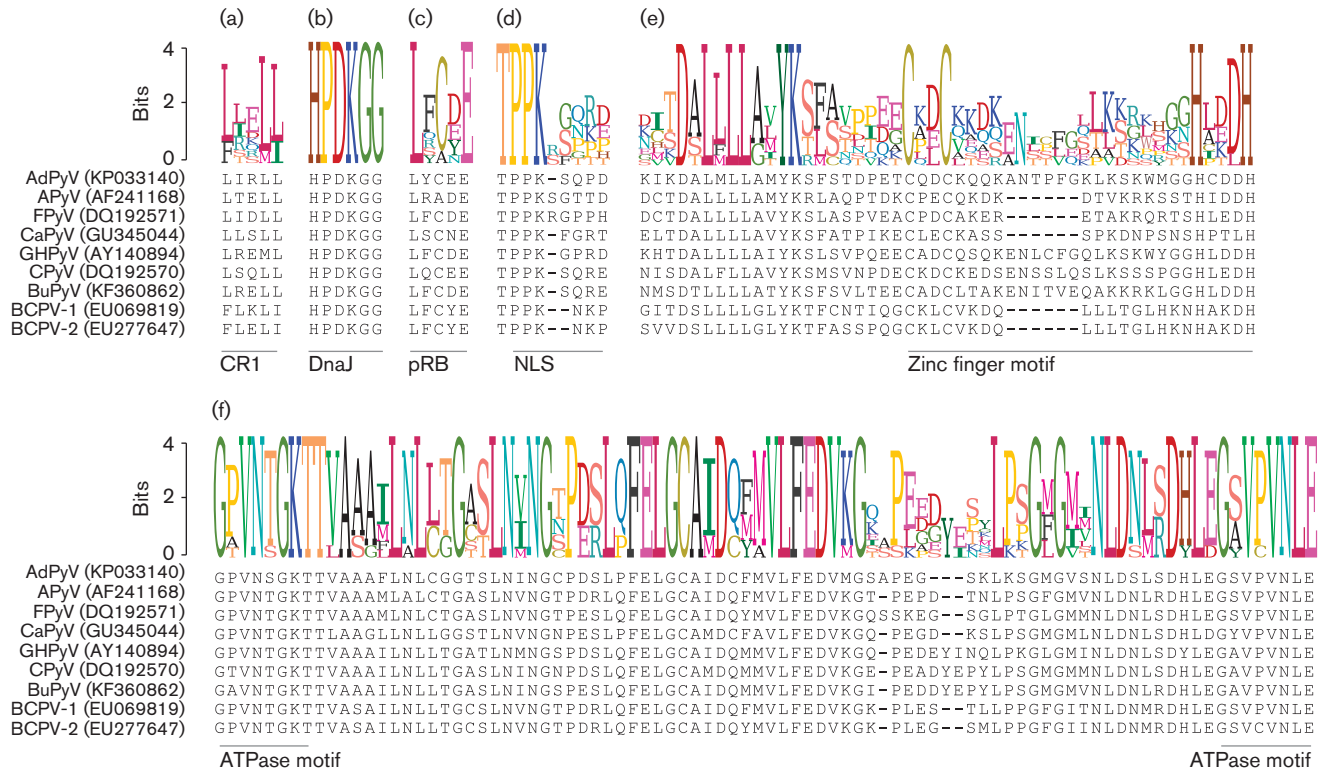


Fig. 3. Conserved CR1 (a), DnaJ (b), pRB (c), NLS (d), zinc-finger (e) and ATPase (f) motifs identified in the T-Ag of the avipolyomaviruses. The sequence log illustration is shown above the motifs to highlight the conserved motif sequences. Gaps in the alignments are denoted by dashes. See Table S1 for abbreviations.

zinc-finger motif (CQDCKQQKANTPFGKLSKWMGG-HCDDH) and ATPase motifs (GPVNSKT and GSPVNLE), which are all relatively conserved among all polyomaviruses (Ehlers & Moens, 2014) and BCPVs (Woolford *et al.*, 2007) (Fig. 3). The consensus CXCX₂C protein phosphate 2A binding domain that is found in almost all mammalian t-Ags was not found to be encoded by AdPyV. In the VP1:VP2 concatenated amino acid sequence ML phylogenetic tree, the avipolyomavirus sequences are nested within those of the orthopolyomaviruses; VP1:VP2 of the wukipolyomaviruses are the most divergent; hence the proposal by John *et al.* (2011) to establish a new genus for these viruses. Nonetheless, the VP1 of AdPyV shared the highest pairwise amino acid identity (58%) with that of butcherbird polyomavirus and goose hemorrhagic polyomavirus (GHPyV), whereas the VP2 was most closely related to GHPyV (42%) (Fig. 1).

Based on the ML phylogenetic analysis of the T-Ag coupled with pairwise identities of the ORFs, AdPyV represents a new species of avipolyomaviruses. As highlighted by Varsani *et al.* (2014) for the *P. adeliae* papillomavirus, we are confident that AdPyV is associated with Adélie penguins and that the chances of contamination by the South Polar skua (*Stercorarius maccormicki*; preys on Adélie penguin eggs and chicks), the only other bird in the subcolony, are extremely slim; the skua do not nest within the faecal trays, are chased away by the penguins, are outnumbered by the penguins by >200:1, and in fact one skua pair defends ~1000 nests, and the tray, within its territory. This report expands our current knowledge of the host range of avipolyomaviruses and to the best of our knowledge this is the first report of a polyomavirus associated with penguins. The identification of this novel avipolyomavirus will enable us to design specific PCR probes to determine its prevalence and host range, given that this information is unknown; furthermore, complete genomes can be recovered from samples that test positive to determine viral diversity.

To the best of our knowledge, this is one of the five genomes of viruses associated with Antarctic animals to be characterized to date; previously identified viral genomes include those of a *P. adeliae* papillomavirus and an avian influenza virus subtype H11N2 from Adélie penguins (Hurt *et al.*, 2014; Varsani *et al.*, 2014) and adenoviruses from South Polar skuas and chinstrap penguins (*Pygoscelis antarctica*) (Lee *et al.*, 2014; Park *et al.*, 2012). This highlights the poor knowledge of viruses associated with Antarctic animals. It is worth noting that this poor knowledge of viruses in Antarctica goes beyond just Antarctic animals. Only a handful of other complete viral genomes have been characterized from soil sampled in the McMurdo Dry Valleys (Meiring *et al.*, 2012; Swanson *et al.*, 2012) and fresh water ponds (López-Bueno *et al.*, 2009; Zawar-Reza *et al.*, 2014).

The emergence and re-emergence of viruses is a major problem that is being driven by habitat and climate change, which can cause changes in the movement and behaviour

of animals and can indirectly result in increased contact with pathogen reservoirs. With this in mind, it is essential to increase our knowledge of viruses circulating in the Antarctic in order to identify pathogens that may pose significant threats to Antarctic animals.

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