A novel virus detected in papillomas and carcinomas of the endangered western barred bandicoot (Perameles bougainville) exhibits genomic features of both the Papillomaviridae and Polyomaviridae

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A novel virus detected in papillomas and carcinomas of the endangered western barred bandicoot (*Perameles bougainville*) exhibits genomic features of both the *Papillomaviridae* and *Polyomaviridae*

Running title: The *Perameles bougainville* papillomavirus type 1.

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ABSTRACT

Conservation efforts to prevent the extinction of the endangered western barred bandicoot (*Perameles bougainville*) are currently hindered by a progressively debilitating cutaneous and muco-cutaneous papillomatosis and carcinomatosis syndrome observed in captive and wild populations. In this study we have detected a novel virus, tentatively designated the *Perameles bougainville* papillomavirus type 1 (PbPV1), in lesional tissue from affected western barred bandicoots using multiply primed rolling circle amplification (RCA) and PCR with cutaneotropic papillomavirus primer pairs FAP59/ FAP64 and AR-L1F8/AR-L1R9. Sequencing of the PbPV1 genome revealed a novel prototype virus exhibiting genomic properties of both the *Papillomaviridae* and the *Polyomaviridae*. Papillomaviral properties included a large genome size (~7.3kb) and the presence of ORFs encoding canonical L1 and L2 structural proteins. The genomic organization in which structural and non-structural proteins were encoded on different strands of the double stranded genome, and the presence of ORFs encoding the non-structural proteins large T and small t antigen were on the other hand typical polyomaviral features. PbPV1 may represent the first member of a novel virus family, descended from a common ancestor of the papilloma- and polyomaviruses recognized today. Alternatively it may represent the product of ancient recombination between members of these two virus families. The discovery of this virus could have implications for the current taxonomic classification of *Papillomaviridae* and *Polyomaviridae*, and can provide further insight into the evolution of these ancient virus families.

INTRODUCTION

The western barred bandicoot (WBB), *Perameles bougainville*, is an endangered Australian marsupial that was once widespread across western and southern Australia. Now extinct on the mainland, wild populations are known only to exist on Bernier and Dorre Islands, in the World Heritage Area of Shark Bay, Western Australia (40, 44, 45). Conservation efforts to prevent the extinction of the WBB are currently hampered by a progressively debilitating cutaneous and muco-cutaneous papillomatosis and carcinomatosis syndrome observed in captive and wild individuals. Lesions appear as irregular thickenings and masses over the skin of the digits, body, pouch and muco-cutaneous junctions of the lips and conjunctiva. Histological, ultrastructural and immunohistochemical features of lesions support the involvement of a papilloma- or polyomavirus in the pathogenesis of this disease (56).

Papillomaviruses (PVs) are transmissible epitheliotropic and species-specific viruses that typically cause excessive irregular proliferation of cutaneous and mucosal epithelia in humans and many mammalian and avian species (27, 48). These viruses exhibit a range of pathogenicity and have been associated with both benign and malignant disease (4, 46, 47, 58). Like PVs, polyomaviruses (PyVs) infect humans and a variety of mammalian and avian species, with fourteen PyV types completely genomically characterized to date (15, 25, 34). The mammalian PyVs display a narrow host range and do not productively infect other species. These viruses typically cause apathogenic subclinical infections in their natural and immunocompetent hosts, but may cause severe disease in the immunocompromised or can cause tumor formation when they are introduced to an unnatural host (9, 29, 41). The hamster PyV (HaPyV) has the ability to naturally infect hair follicle keratinocytes and induce cutaneous epitheliomas (17, 43). In contrast to the mammalian PyVs which are characterized by subclinical persistent infection, the avian PyVs Goose hemorrhagic PyV (GHPyV) and Budgerigar fledgling disease PyV (BFPyV), and possibly the finch (FPyV) and crow (CPyV) PyVs, are associated with fatal disease in birds (20, 25, 31).

PVs and PyVs were previously considered subfamilies of the *Papovaviridae* family. Both are small non-enveloped DNA viruses comprising a single double-stranded circular DNA genome within an icosahedral capsid. Furthermore they share the ability to cause malignant neoplasia, and use similar strategies to take control of the host cell DNA replication and transcription processes. As it was later recognized that the two virus groups have significantly different genome sizes and organization, and encode a different number and type of structural and non-structural proteins, they are currently recognized as two separate virus families by the International Committee on the Taxonomy of Viruses (11, 22). The two families can also be distinguished by the presence of conserved family-specific epitopes in their major capsid proteins (23).

Putative PV sequences have been amplified from the skin of Australian marsupial species, namely from a cutaneous papilloma of a common brush-tail possum (*Trichosurus vulpeca*) (35) and from the healthy skin of koalas (*Phascolarctos cinereus*) and an eastern grey kangaroo (*Macropus giganteus*) (3). However, complete genomic sequences were not deduced in these studies, rather sequencing was restricted to that of amplicons obtained from the L1 region of each virus using degenerate primer PCR. Here we present the genomic sequence of a novel prototype virus isolated from a marsupial species exhibiting genomic properties of both the *Papillomaviridae* and the *Polyomaviridae*, which we have tentatively designated *Perameles bougainville* papillomavirus type 1 (PbPV1). PbPV1 was amplified from lesional tissue and from superficial skin swabs taken from the surface of lesions from western barred bandicoots affected by a papillomatosis and carcinomatosis syndrome.

MATERIAL AND METHODS

Clinical material. Between 2000 and 2007, cutaneous and muco-cutaneous lesional and non-lesional tissue samples from 32 WBBs affected by a papillomatosis and carcinomatosis syndrome (56) and two unaffected individuals were received by the Pathology Section at the Murdoch

University School of Veterinary and Biomedical Sciences. Tissues collected from 19 WBBs were divided in two, one half frozen for molecular techniques and the other half collected into 10% neutral buffered formalin for histological evaluation. Submissions from the remaining 13 individuals were collected at a referring wildlife breeding centre and submitted as formalin-fixed tissues only. Tissue samples were classified histologically in a previous study as either unaffected (normal) epithelia, papillomatous hyperplasia, carcinoma *in situ*, squamous cell carcinoma or adenocarcinoma (56). All submissions were received from captive populations of WBBs held at either Kanyana Wildlife Rehabilitation Center (KWRC), Perth, WA; Dryandra Woodland captive breeding facility, Narrogin, WA; or the Peron Captive Breeding Center (PCBC), Denham, Shark Bay, WA.

Collection of superficial skin swabs. Superficial skin swabs collected for the purpose of viral DNA detection were taken from 73 WBB examined during field monitoring activities carried out between 2005 and 2007 using sterile 0.9% NaCl soaked cotton tip swabs as per Martens and others (2001) and Antonssen and others (2002) (2, 30). The number and origin of WBB examined during this study and the number of individuals with clinical evidence of the papillomatosis and carcinomatosis syndrome is presented in Table 1. Sites swabbed from each individual included cutaneous or muco-cutaneous lesions if present, and the skin of the lateral flank, lip commissure and feet and the conjunctival epithelium. Swabs were rubbed against the skin or lesion surface six to ten times and then placed into sterile saline within a 1.5ml microcentrifuge tube and stored at -20 °C. In the laboratory, microcentrifuge tubes containing the cotton tip swabbed were thawed and vortexed for 30 seconds. Each sample was spun down briefly, following which the cotton tipped swab was removed and discarded using sterile forceps and the remaining suspension was stored at -20 °C until further testing.

DNA extraction from skin lesions. Sixty three fresh tissues samples from 19 affected and 2 unaffected individuals, and 13 formalin-fixed samples from 13 affected individuals were selected for molecular techniques. Total genomic DNA was isolated from 25 mg of each tissue sample using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's protocol for either fresh tissue or formalin fixed tissues, respectively.

Multiply primed rolling circle amplification. Multiply primed rolling circle amplification was performed on 13 lesional extracts from six affected WBB using the TempliPhiTM 100 Amplification Kit (Amersham Biosciences) using a modified protocol optimized for the amplification of viral circular DNA genomes (39). One µl of extracted WBB lesional DNA (containing 1-5 ug of total DNA), 2 ng of pUC18 (positive control) or water (negative control) were transferred to a 0.5 ml tube with 5 µl of TempliPhi Sample Buffer, denatured at 95°C for 3 minutes and afterwards placed on ice. For each sample, a premix was prepared on ice by mixing 5 µl TempliPhi Reaction Buffer, 0.2 µl TempliPhi enzyme mix containing the Phi 29 polymerase, and an extra 450 µM of each dNTP. After vortexing, 5 µl of the premix was added to each of the cooled samples, reactions were gently mixed and incubated overnight at 30°C. Following overnight incubation, the reactions were heated to 65°C for 10 minutes to inactivate the ϕ29 DNA polymerase and then stored at –20°C.

Restriction enzyme analysis. To investigate whether viral DNA had been amplified in the reaction, 2 µl of the RCA product from each sample was digested with 10 units of *Bam*HI or *Sal*I (Promega), respectively, in a total volume of 10 µl for each reaction. Each digest was separated via gel electrophoresis in a 0.8% agarose gel and visualized through ethidium bromide staining to check for the presence of a DNA band consistent with full-length papillomaviral DNA (circa 8 kb), or multiple bands with sizes adding up to this length.

DNA transformation and cloning. Digestion of the RCA products from WBBs 1-4 (Table 2) with either *Bam*HI or *Sal*I resulted in one DNA fragment of approximately 7.5 kb, respectively. RCA products obtained from the lip lesion of WBB 2 and the eyelid lesion of WBB 3 were selected for cloning. Five µl of each RCA product was digested with 40 units of *Bam*HI or *Sal*I respectively and run on a 0.8% agarose gel, after which each ~7.5 kb fragment was cut from the gel and the DNA was extracted from the gel slice using the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's protocol. Each fragment was then ligated into dephosphorylated BamHI-cut or SalI-cut pUC18 using the Roche Rapid DNA ligation kit (Roche) according to the manufacturer's protocol. After transformation of One Shot MAX Efficiency DH5α-T1R competent cells (Invitrogen) with the ligation product, the bacteria were incubated for blue-white colony screening on agar plates containing X-gal, and white colonies were checked by BamHI and SalI digestion of miniprep DNA. Minipreps were performed using the QIAprep Miniprep Kit (Qiagen) according to the manufacturer's protocol.

Plasmid sequencing. Multiple clones containing the *Bam*HI-cut ~7.5 kb DNA fragment or *Sal*I-cut ~7.5 kb DNA fragment obtained by digestion of RCA products from WBB 2's lip lesion and WBB 3's eyelid lesion respectively were selected for sequencing. The complete genome of the western barred bandicoot (*Perameles bougainville*) papillomavirus type 1 (PbPV1) was determined by primer-walking sequencing of cloned DNA fragments, starting from the universal primers in the multiple cloning site of pUC18. In total, 21 primers were used to cover the complete genome on both strands. Each clone was sequenced three times in entirety. Sequencing of the purified plasmids was carried out using the dideoxynucleotide chain termination method (42). Sequence was determined using an ABI Prism Applied Biosystems 377 DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.) at the State Agriculture and Biotechnology Centre, Perth, Western Australia. Chromatogram sequencing files were edited using Chromas Lite version 2.0

(Technelysium Pty Ltd, Helensvale, Australia) and contigs were assembled using BioEdit v7.0.5 (URL: http://www.mbio.ncsu.edu/BioEdit/).

Long template PCR. Long range PCR primers, designed from PbPV1 sequence data obtained from cloning and sequencing of RCA products, were applied directly to the lesional DNA extract from WBB 1 to sequence parts of the putative PbPV1 genome independently of RCA. Primers were chosen to produce amplicons encompassing the regions linking the PV-like and PyV-like ORFs of the PbPV1 genome, in order to confirm the occurrence of both PV- and PyV-like ORFs in a single viral genome. Primer pairs used were LTPCR-LTF1 in large T (5'-GCCTCTAGAAAGCACATTATTACCTCCAGGGTTTGG-3') and LTPCR-L1R1 in L1 (5'-ATGTAAAGTCCCTCTCAAGCCAGAAGTCCTAGC-3') for amplification of a fragment of 2130 nucleotides, linking the large T and L1 ORFs of PbPV1, and LTPCR-L2F2 in L2 (5'-CACCACCTCTACCTGTTCCTATACCTAATCCACC-3') and LTPCR-STR2 in small t (5'-CATCACCTTCACTCTCGTAGCAGAACAGATCTTCC-3') for a second fragment of 1259 nucleotides, encompassing the region that connects the L2 ORF with the small t ORF. Long template PCR was performed with the Expand Long Template PCR System (Roche Diagnostics) according to the manufacturer's instructions. The amplification profile consisted of 2 minutes denaturation at 94 °C, followed by 10 cycles of 10 seconds denaturation at 94 °C, 30 seconds annealing at 63 °C and 4 minutes elongation at 68 °C. This was followed by 25 cycles of 10 seconds denaturation at 94 °C, 30 seconds annealing at 63 °C and 4 minutes elongation at 68 °C with an increase in elongation time by 20 additional seconds each cycle. After a final elongation step of 7 minutes at 68 °C the samples were then cooled to 4 °C. The PCR products were gel-purified by using the QIAquick Gel Extraction Kit (Qiagen), followed by direct sequencing of the purified amplicons with nested sequencing primers. Primers LTF1 (5'-TCACCAAAGCTCATAAAGCAG-3') and L1R1 (5'-GAGAAAGTTCTTGTATCAGAGC-3') were used to determine a 1531 nucleotide strectch of the first amplicon, and with primers L2F2 (5'-

CTGTTCCTATACCTAATCCACC-3') and STR2 (5'-GCCATCACAAGTATCACAACC-3') 1051 nucleotides of the second amplicon were sequenced.

DNA sequence submission. The nucleotide sequence data reported in this article was deposited in GenBank using the National Center for Biotechnology Information (NCBI, Bethesda, MD) BankIt v3.0 submission tool (http://www3.ncbi.nlm.nih.gov/BankIt/) under accession number EU069819.

PCR detection using degenerate PV-specific primers. Polymerase chain reactions with degenerate cutaneotropic PV-specific primers were performed on RCA products, tissue DNA extracts and skin swab suspensions with the degenerate primer pairs AR-L1F8/AR-L1R9 (37) or FAP59/FAP64 (14). PCR was carried out in a total volume of 50 µl, containing 200 µM of each dNTP, 0.75 µM of forward and reverse primer, 1 U of Taq DNA polymerase, 1.5 mM $MgCl_2$ [pH 8.5] and 1x DNA Polymerase Reaction Buffer, with 2 µl of the 1:10 diluted RCA product, extracted lesional DNA or skin swab suspension as template. PCR reagents were supplied by Fisher Biotech Australia, Perth, Western Australia or Perkin Elmer/Roche Molecular Systems, Belgium. PCR was carried out in an automated thermocycler (Perkin Elmer Gene Amp PCR System 2400, Foster City, CA, USA) programmed for block temperatures, using the following parameters: 10 minutes at 94 °C and then 45 cycles of 1.5 minutes at 94 °C, 1.5 minutes at 50 °C and 1.5 minutes at 72 °C, followed by 5 minutes at 72 °C. The PCR products were purified through 2% agarose gel electrophoresis, PV-specific amplicons were extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen) and then sequenced with the same degenerate primers as used for PCR. The sequence of the PCR products was determined and edited as described above for plasmids. Similarity searches were performed using the NCBI BLAST (Basic Local Alignment Search Tool) server (version 2.2.13) on the NIH genetic sequence database GenBank (1).

PCR detection using PbPV1-specific primers. Polymerase chain reactions with PbPV1-specific primers were performed on RCA products, tissue DNA extracts and skin swabs suspensions with the primer pairs LWL1F (5'-GAGGAGGGACATCAGGTGAC-3') / LWL1R (5'-ATTGTTTTGCCCAGTTGCTC-3') designed within the PbPV1 L1 ORF to produce a 176 nt amplicon. PCR was carried out in a total volume of 25 µl, containing 200 µM of each dNTP, 0.3 µM of forward and reverse primer, 0.5 U of Taq DNA polymerase, 1.5 mM $MgCl_2$ [pH 8.5] and 1x DNA Polymerase Reaction Buffer, with 2 µl of the 1:10 diluted RCA product, extracted lesional DNA or skin swab suspension as template. PCR reagents were supplied by Fisher Biotech Australia, Perth, Western Australia. PCR was carried out in an automated thermocycler (Perkin Elmer Gene Amp PCR System 2400, Foster City, CA, USA) programmed for block temperatures, using the following parameters: 5 minutes at 94 °C and then 30 cycles of 30 seconds at 94 °C, 30 seconds at 55 °C and 30 seconds at 72 °C, followed by 7 minutes at 72 °C. The PCR products were purified through 2% agarose gel electrophoresis, extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen), and sequenced with the same primers as used for PCR. The sequence of the PCR products was determined and edited as described above for plasmids.

DNA and protein sequence analysis. The putative open reading frames (ORFs) were predicted using the ORF Finder tool on the NCBI server of the National Institutes of Health (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Similarity searches were performed using the NCBI BLAST (Basic Local Alignment Search Tool) server (version 2.2.13) on the NIH genetic sequence database GenBank (1). Protein sequence similarities between PbPV1 and known papillomaviruses and polyomaviruses were investigated by pairwise sequence alignments using the GAP-program on the Sequence Analysis Server at Michigan Technological University (http://genome.cs.mtu.edu/align/align.html).

Phylogenetic analysis. To determine the evolutionary relationship of the PbPV1 sequence to other PV types, phylogenetic analysis was performed on the nucleotide sequences of the L1 and L2 ORFs of PbPV1, the 46 PV types that are classified as type species of the different PV genera and species, and 7 recently identified and currently unclassified PV types. The L1 and L2 protein coding regions of these sequences were aligned at the amino acid level with ClustalW (51) using the BLOSUM protein weight matrix in the DAMBE software package version 4.2.13 (57). This amino acid alignment was then used as a template for aligning the corresponding nucleotide sequences in DAMBE. The nt alignments were corrected manually in the GeneDoc Multiple Sequence Alignment Editor and Shading Utility software package version 2.6.002 (33), retaining only the unambiguously aligned parts. Based on these alignments, phylogenetic trees were constructed for L1 and L2 separately, as well as for a concatenated alignment of both ORFs, by using the neighbor-joining method and with calculation of bootstrap support values for 10,000 replicates by the neighbor-joining method in MEGA version 3.1 (26).

For the PbPV1 large T antigen (LT Ag), the evolutionary relationship to PyVs was investigated. For this analysis, 13 PyV species for which a complete genomic sequence was available in GenBank were included. Because the intron sequence within the PbPV1 LT Ag gene could not be confidently identified, the analysis was performed on the complete LT Ag genomic sequences, so including intron sequences. The LT Ag nucleotide sequences were aligned with the ClustalW algorithm (51) implemented in the DAMBE software package version 4.2.13 (57). Divergent regions and positions in which the sequences were not unambiguously aligned were removed from the alignment by using the Gblocks computer program version 0.91b (7). Based on this corrected alignment, a neighbor joining phylogenetic tree was constructed in the same way as described above.

RESULTS

Detection of a novel papillomavirus by using multiply primed rolling circle amplification, degenerate primer PCR and long template PCR. *Bam*HI or *Sal*I digestion of RCA products

amplified from five lesional biopsies collected from WBBs 1-4 (Table 2) revealed one ~7.5 kb band in each sample consistent with full length PV DNA (data not shown). RCA products from WBBs 2 and 3 were cloned and sequencing revealed the complete genome of a novel virus had been amplified. A 450 bp DNA fragment was amplified with the FAP59/64 primer pair in both the lesional DNA extracts and the RCA products and subsequent sequencing of these bands demonstrated they had identical nucleotide sequences. PCR on total lesional DNA extracts with the AR-L1F8/AR-L1R9 primer pair generated an amplicon of 700 bp. A BLASTX query with both the 450 bp and 700 bp sequences showed that these amplicons were homologous to papillomaviral L1 sequences, and that they were not identical to any of the previously identified PV types. Long range template PCR was applied to lesional DNA extracts independently of RCA. Amplicons were generated that were identical to PbPV1 sequences obtained by sequencing of cloned RCA products and encompassed sequences from both the early and late ORFs, revealing that early and late region ORFs were continuous with one another on template DNA (PbPV1 genome) and the sequences were identical to those retrieved from the cloned RCA products.

Analysis of PbPV1 complete genomic sequence and deduced amino acid sequences. The newly identified virus was tentatively named the *Perameles bougainville* papillomavirus type 1 (PbPV1) and its complete genome counted 7295 bp, with a GC content of 37.4% (GenBank accession number EU069819). The genomes of the novel PV amplified from the lip lesion of WBB 2 and an eyelid lesion of WBB 3 were sequenced in entirety and found to be identical. ORF analysis and BLASTX searches predicted the presence of the two classical papillomaviral late (L) protein ORFs, and two polyomaviral T antigen-like early region ORFs. Putative T antigens were encoded on the opposing strand as the late protein ORFs (Fig. 1A).

The genome of PbPV1 exhibited two ORFs encoding proteins demonstrating greatest similarity to the PV major (L1) and minor (L2) capsid proteins respectively. The putative L1 ORF was 1518 bp

in length (nt 2240 – 3757) and the putative L2 1409 bp (nt 818 to 2227) in length. The sequence similarity between the respective L1 and L2 ORFs of PbPV1 and HPV1a (a benign cutaneous PV of the μ-PV genus; NC_001356), HPV5 (an epidermodysplasia verruciformis-associated PV of the β-PV genus; NC_001531), HPV16 (a mucosal high-risk PV of the α-PV genus; NC_001526), HPV6 (a mucosal low-risk PV of the α-PV genus; X00203) and bovine BPV1 (a fibropapillomavirus of the δ-PV genus; X02346) was investigated by pairwise alignments performed at the amino acid level. The similarity of the putative PbPV1 L1 and L2 sequences to those of the PV types listed above ranged between 47-51% and 26-33% respectively (Table 3). The PbPV1 late ORFs did not demonstrate any similarity to classical polyomavirus capsid protein encoding ORFs VP1, VP2 or VP3.

In addition to the late proteins described above, two PbPV1 ORFs coded for proteins demonstrating high similarity to the avian and mammalian PyV T antigens: a putative large T antigen (nt 7295 – 5070) and putative small t antigen (nt 7295 - 6621). The sequence similarity between the respective large T antigen and small t antigen ORFs of PbPV1 and thirteen known PyVs [Lymphotropic PyV of African Green Monkey (LPyV) NC_004763, Simian virus 40 (SV40) NC_001669, Simian agent 12 (Sa12) NC_007611, Hamster PyV (HaPyV) NC_001663, Mouse PyV (MPyV) NC_001515, Kilham strain of mouse PyV (MptV) NC_001505, Bovine PyV (BPyV) NC_001442, JC virus (JCV) NC_001699, BK virus (BKV) NC_001538, Budgerigar fledgling disease PyV (BFPyV) NC_004764, Goose hemorrhagic PyV (GHPyV) NC_004800, Crow PyV (CPyV) NC_007922, Finch PyV (FPyV) NC_007923] was investigated by pairwise alignments performed at the amino acid level. Due to the dissimilarity of this virus to known PyVs, a putative intron was not able to be predicted within the PbPV1 large T antigen ORF. Therefore alignments were performed using the PbPV1 large T protein sequence predicted from the unspliced ORF nucleotide sequence. The similarity of the putative PbPV1 large T antigen and small t antigen sequences to those of known PyVs ranged between 19-35% and 0-22%, respectively (Table 4).

Further analysis of these proteins revealed conserved PyV T antigen motifs. A DnaJ domain was located between aa 8-72 of PbPV1 large T antigen and small t antigen proteins, including the conserved hexapeptide HPDKGG (Table 5A), and an ATPase binding domain (GPVNTGKT) was present between aa 543-550. The conserved PyV T antigen LXCXE motif, LFCYE, was found located between aa 78 to 82 within both the putative large T and small t antigens (Table 5B). A C_2H_2 zinc finger motif, common to all PyV T antigens, was found within the putative large T antigen, C-416, C- 419, H-430, H-433 (Table 5C).

The genomic organization of PbPV1 was found to be most similar to that of the *Polyomaviridae*, therefore based on this knowledge a viral regulatory region, containing the origin of replication (*ori*), was predicted to lie between the shared start codon of the T antigens and the start codon of L2 (nt 1-817). Two GAGGC pentanucleotides were located within this region and may represent potential T antigen binding sites. A second non-coding region was also found to lie between the end of L1 and the end of the large T antigen ORFs (nt 3758 – 5069). No recognizable regulatory or promoter elements were identified in this region and BLAST performed with this region did not reveal any similarity to known PVs or PyVs.

Phylogenetic analysis. Since PbPV1 shows similarity to polyomaviral genomes in its early region but has a late region encoding papillomaviral proteins, the evolutionary history of PbPV1 was reconstructed separately for both regions, based on comparison to the polyomaviral large T antigen and the papillomaviral L1 and L2 capsid genes, respectively. A neighbor-joining phylogenetic tree was constructed based on an alignment of the large T antigen sequences of PbPV1 and 13 members of the *Polyomaviridae* (Fig. 2). This tree showed two major branches, containing the PyVs infecting Eutherian mammals in one cluster, and those infecting birds in the other. PbPV1 did not belong to either cluster but branched off directly from the root between the mammalian (Eutherian) and avian

PyVs, which is in agreement with the WBB host species belonging to the marsupials (Metatheria infraclass of mammals), in which no PyV has been isolated thus far. This corroborates a scenario in which the PbPV1 early region would have branched off of the PyV evolutionary tree very early in evolution.

An L1 neighbor-joining phylogenetic tree was constructed based on alignment of the L1 ORF of PbPV1 and 53 members of the *Papillomaviridae* (Fig. 3). This L1 tree clustered the PVs in the previously defined genera, and the nodes joining different PVs that belong to the same genus were supported by high bootstrap values. This tree indicated that the WBB virus was most closely related to the members of the β-PV genus, but it branched off very close to the root of the common branch of this genus and this clustering was only supported by a low bootstrap value (66%). In a neighbor-joining phylogenetic tree based on an L2 sequence alignment of the same papillomaviruses, PbPV1 did not share a common branch with the members of the β-PV genus. Instead, it originated near the root of the evolutionary tree in a common branch with the γ-PV and the rodent PVs HaOPV, McPV2 and MmPV (data not shown). Again, this clustering showed only low bootstrap support.

Screening of WBB papillomas, carcinomas and non-lesional epithelia by PCR. PbPV1 was amplified from the skin lesions of 18/19 (94.7%) affected WBB for whom DNA was extracted from fresh lesional tissue. In affected WBB for whom only formalin-fixed tissues were available, PbPV1 was detected in skin lesions from 4/13 (30.7%) WBBs. Histological classification and PCR results of lesions screened for PbPV1 DNA are presented in Tables 6A-B. Both FAP59/64 and PbPV1-specific primers pairs were utilized in tissue screening and sequencing of amplicons indicated that only viral sequences identical to PbPV1 were amplified from these tissue extracts. No viral DNA was amplified from cutaneous and mucosal tissue extracts from the two unaffected WBB.

Screening of skin swabs taken from WBB in the field. Superficial skin swabs taken from the surface of lesions from each of the eight WBB found to be clinically affected by the papillomatosis and carcinomatosis syndrome tested positively for PbPV1 DNA. Both FAP59/64 and PbPV1-specific primer pairs were utilized and sequencing of amplicons indicated that only viral sequences identical to PbPV1 were amplified from skin swab suspensions. Affected WBB testing positive for PbPV1 originated from a wild population on Bernier Island (n=2) and from a captive breeding colony at Dryandra Woodland (n=6). PbPV1 was not detected in skin swab suspensions taken from the surface of normal cutaneous and mucosal epithelia in the 65 clinically normal WBBs examined in the field.

DISCUSSION

PbPV1 was found to possess a double-stranded circular DNA genome 7295 bp in length which is consistent with members of the *Papillomaviridae*. However, its genomic organization comprising ORFs encoding structural proteins located on one strand and ORFs encoding non-structural proteins on the opposite strand, is characteristic of the *Polyomaviridae* and unlike that of the *Papillomaviridae*, in which all ORFs are located on (and genes are subsequently transcribed from) one DNA strand only (9, 22).

The putative PbPV1 late region codes for PV-like major (L1) and minor (L2) capsid protein genes. The L1 ORF is the most conserved region among different PV types and the currently accepted classification of these viruses is based upon similarities in this region (11). The PbPV1 L1 shared less than 60% identity to the L1 of other PVs, therefore on the basis of this sequence analysis alone it cannot be attributed to one of the existing PV genera. However, the PbPV1 L1 sequence was sufficiently similar to that of other cutaneous PV types to allow detection with degenerate primer pairs designed to detect a broad range of cutaneotropic PV types.

Two predicted PbPV1 ORFs encoded proteins that are most similar to PyV large T and small t antigens. The T antigens are multifunctional proteins that perform a diverse array of activities, including alteration and recruitment of specific host cell proteins to participate in virus production, blockade of cellular antiviral defense systems and direct participation in viral replication (5). The transforming properties of the T antigens are mediated through their direct physical association with cellular target proteins, such as the retinoblastoma protein family of tumor suppressors Rb, p107 and p130 (large T antigen), components of the cellular signal transduction network (middle T antigen) and cellular protein phosphatase PP2A (small t antigen) (5).

PyV T antigens are encoded by a common precursor mRNA that is differentially spliced to create multiple monocistronic mature mRNAs. For example SV40 expresses three such mRNAs, one each for the large T antigen, small t antigen and tiny t antigen, whereas murine PyV expresses four mRNAs, one each for large, middle, small and tiny t antigens (5, 9). Due to the dissimilarity of the PbPV1 genome to known PyVs, it was not possible to predict T antigen mRNA splice junctions from this sequence analysis alone. Examination of PbPV1 T antigen proteins for conserved PyV motifs was therefore performed on translated unspliced mRNAs predicted in the ORF analysis.

A DnaJ domain was identified in the putative PbPV1 small t and large T antigen sequences. In previously characterized PyVs the small t and large T antigens share the amino-terminal portion of the early domain, inclusive of the DnaJ domain, due to the splicing pattern of early viral mRNAs (36). The DnaJ domain of the T antigens plays an essential role in virion assembly, virus DNA replication, transcriptional control and oncogenic transformation (21), and with the exception of the bovine PyV large T antigen, all characterized PyV large T antigens and small t antigens contain the conserved HPDKGG motif (36). The PyV ATPase binding domain, GP(V/I)(N/D)XGKT (55), was also identified within PbPV1 T antigen sequences. This is the most highly conserved domain among PyV types. Sequences important for complex formation with the cellular p53 protein also lie within

this domain (36). A large T antigen consensus motif LXCXE, required for binding to the tumor suppressor protein Rb and to two structurally related proteins, p107 and p130 (21), was identified in PbPV1 as LFCYE. PbPV1 early ORFs did not demonstrate any similarity to classical PV early region ORFs such as E1, E2, E6 or E7 at either a nucleotide or amino acid level, except for the presence of the pRB binding motif, which is a common feature of both PyV large T and PV E7 proteins.

Based on the PyV-like genomic organization of PbPV1, a viral regulatory region and origin of replication (*ori*) was predicted to lie between the start of L2 and the start of the T antigens. The regulatory region of PyVs is located between the early and late regions and comprises the origin of replication (*ori*), the TATA box, T antigen binding sites, cellular transcription factor binding sites, and bidirectional promoter and enhancer for transcription of early and late genes (9). Transcription extends bidirectionally from initiation sites near the origin, with early and late mRNAs transcribed from opposite strands of the viral genome. The *ori* of most characterized PyVs consists of multiple copies of the GAGGC pentanucleotide, flanked by A/T-rich sequences on the late side and an imperfect palindrome on the early side, with the exception of BFDV and GHDV which do not contain any GAGGC pentanucleotides within the regulatory region (6, 36). In mammalian PyVs, the large T antigen binds to this GAGGC pentanucleotide (52). In avian PyVs, however, the binding sequence for the large T antigen has been identified as the palindromic sequence TCC $(A/T)_6$ GGA or similar (25, 28), of which related sequences were not identified in the PbPV1 sequence. Two potential GAGGC large T antigen binding sites were identified in the putative PbPV1 viral regulatory region.

The NCR region of PVs typically lies between the stop codon of L1 and the start codon of E6. PVs usually contain an E1-recognition site flanked by two E2 binding sites (with the consensus sequence $ACCN_6GGT$), for binding of an E1/E2 complex in order to activate the origin of replication. These

sites were not identified within the PbPV1 viral regulatory region, which was not unexpected since PbPV1 does not appear to encode any proteins demonstrating similarity to papillomaviral E1 or E2.

A second non-coding region was located between the ends of the PbPV1 L1 and large T antigen ORFs, however no significant homology to known PyV or PV types was found in the region and we were unable to identify regulatory or promoter elements. A second non-coding region located between the end of the early and the beginning of the late protein region has also been characterized in the λ PV types, including the feline PVs, canine oral PV (COPV) and the *Procyon lotor* PV (PlPV1) (13, 37, 38, 49, 50) and in the deer PV (δ PV) (19). It has been postulated that this region may have arisen though an ancient integration event, and may have structural or functional importance that is yet unknown (38).

Due to novel nature of this viral genome, concerns were initially raised that there may be a dual PV and PyV infection in WBB lesional tissue, or that contamination of WBB DNA extracts with either PV or PyV viral DNA may have occurred in the laboratory, and therefore this unique hybrid-like PV-PyV genome may in fact be an artifact of the RCA technique. Sequencing of long range template PCR products amplified directly from extracted lesional DNA indicated that the PV-like and PyV-like sequences were continuous with one another on the PbPV1 template DNA. In addition, the partial sequences of PbPV1 generated using long template PCR independently of RCA were found to be identical to the PbPV1 sequence obtained from cloned RCA products. The findings that the same size 7.3 kb genome was detected in biopsies from four different individuals, genomes from two of these reactions had identical sequences, and that these sequences were unlike previously characterized PVs and PyVs were also supportive of the existence of this novel genome.

PbPV1 was detectable in 94.7% of individuals from whom fresh lesional tissue extracts were screened, 30.8% of individuals for whom formalin fixed tissues extracts were screened and 100% of

individuals displaying clinical evidence of the papillomatosis and carcinomatosis syndrome screened by a skin swabbing technique. Amplification of PbPV1 from DNA extracted from formalin-fixed lesions may not have occurred as readily due to deleterious effects on DNA quality and quantity that might be caused by formalin-fixation of tissues (16). PbPV1 was detected in the vast majority of epithelial lesions consistent with the papillomatosis and carcinomatosis syndrome including papillomatous hyperplasia, carcinoma *in situ* and squamous cell carcinomas, however, not in the single case of adenocarcinoma. PbPV1 was not detected in tissue samples or skin swabs taken from clinically normal individuals. It appears that PbPV1 is only detectable in individuals displaying skin lesions and therefore unlikely to be a commensal of normal WBB skin. No viral sequences other than PbPV1 were amplified using broad spectrum cutaneotropic PV and PbPV-specific primer pairs. In addition to the results presented in this study, screening of WBB lesions using MY09/11 primer pairs designed to amplify a broad range of mucosotropic PV types (18) and a broad spectrum nested PCR technique for the amplification of PyVs (24) was also performed but failed to amplify PV or PyV genomic DNA, respectively (data not shown). These results are supportive of the hypothesis that PbPV1 is a necessary factor for development of the papillomatosis and carcinomatosis syndrome, and it appears at this stage that only one virus type is associated with this disease in the WBB.

The observed patterns of disease in captive populations are suggestive that disease is possibly transmitted from affected to unaffected WBB through direct contact such as when fighting and mating (56). Earlier studies to examine the transmissibility of this disease using an immunodeficient mouse model were largely unsuccessful and no experimental work has yet been performed to ascertain the infectivity of the PbPV1 genome.

PbPV1, detected in cutaneous and muco-cutaneous lesions from the endangered western barred bandicoot, represents a novel prototype virus possessing genomic characteristics of both the

Polyomaviridae and *Papillomaviridae*; specifically possessing the genome size and encoding the structural proteins of the *Papillomaviridae*, and encoding the non-structural proteins and exhibiting the genomic organization of the *Polyomaviridae*. A quandary is created in regards to the taxonomic classification of this virus based on currently accepted classification systems. Over one hundred PV types have been completely characterized in humans alone (11). An additional 40 complete genomes of non-human PVs, retrieved from 31 distinct amniote host species (mammals and birds), are currently available in GenBank. The position, size and function of many of the ORFs are well conserved among the various PV types that have been sequenced and studied thus far (8). This conserved genomic organization provides strong evidence for a monophyletic origin of the extant PVs. Fourteen PyV species have been characterized to date and similarly the many highly conserved regions of PyV genomes support the idea that all known members of the *Polyomaviridae* are descendents of a common ancestor (9).

PbPV1 may have arisen following a recombination event between existent (but yet undiscovered) or ancestral members of the *Papillomaviridae* and *Polyomaviridae* families, in which a PV provided the late region structural genes cassette and a PyV delivered the early region non-structural genes cassette to the recombinant virus. However, although recombination events have been suspected in PVs (32, 54) these are highly uncommon and no evidence has yet been found for recombination among the major PyV clades (10). As these viruses utilize the host cell polymerase during replication, they are relatively stable and slow evolving viruses and evolution is thought to occur mainly through point mutations. PVs and PyVs are also species-specific or show a narrow host range. Various phylogenetic and cophylogenetic studies provide evidence that PVs and PyVs are ancient viruses that originated early in vertebrate evolution and have coevolved and speciated in synchrony with their host species (10, 34, 38, 53). Alignment of papillomavirus L1 sequences inclusive of PbPV1 and the subsequent phylogenetic analysis demonstrates PbPV1 is most closely related to the members of the β-PV genus (containing the Epidermodysplasia verruciformis

associated human PVs). However, it branches off very close to the root of the common branch of this genus (Fig. 3). This seems to be an argument for the virus being the result of recombination between existing or ancient PVs and PyVs rather than being a descendent of the ancient common ancestor of both virus families. This represents the first report of a possible recombination between members of the *Papillomaviridae* and the *Polyomaviridae* families.

Alternatively PbPV1 could be a descendant of a common ancestor to both the modern day *Polyomaviridae* and *Papillomaviridae*. Despite sharing no major nucleotide or amino acid similarity, PVs and PyVs encode proteins sharing similar functions and domains. The actions of PV E7 and PyV large T antigen are similar in the host cell, including induction of cellular DNA synthesis in growth arrested cells and the ability to immortalize cells (21). E7 and the large T antigen share a functional domain, with the consensus motif LXCXE, for binding of the retinoblastoma gene product p105-Rb (12, 21). Therefore, although overall sequence similarity between PVs and PyVs is low, the conservation of small functional domains and exploitation of similar cellular regulatory pathways is suggestive of an ancient common evolutionary origin for both virus families (53). In the large T antigen tree (Fig. 2), PbPV1 appears as a close-to-root sequence, branching off from the root between the mammalian and avian polyomaviruses. In contrast to the L1 analysis, this would be in agreement with the virus being a descendent of a common PV and PyV ancestor, or at least having arisen as long ago as the two major phylogenetic clades of PyVs, so in the earliest evolutionary history of the *Polyomaviridae*.

We have tentatively designated this virus a member of *Papillomaviridae* family for the reason that PbPV1 encodes PV-like structural proteins and exhibits the genome size of the *Papillomaviridae*. In addition, the gross, histological, ultrastructural and immunohistochemical features of the papillomatosis and carcinomatosis syndrome with which this virus has been associated are most consistent with papillomavirus associated skin disease described in other species. However, due to

the novel nature of this virus we propose that it is inappropriate for this virus to be definitively classified as a PV or a PyV, and it may in fact represent the first member of a new family of viruses. We propose its classification as the first member of putative virus family "*Papomaviridae*", where "Pap" represents the encoded pap illomavirus features and "oma" indicating both the poly oma virus features and apparently oncogenic properties of this virus. Not only is the detection of PbPV1 important for conservation efforts to prevent the extinction of the western barred bandicoot, it will surely open discussion concerning theories on the evolution and divergence of virus families previously belonging to *Papovaviridae,* as well as the final classification of this virus.

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389-3402.
2. **Antonsson, A., and N. A. McMillan.** 2002. Healthy skin of many animal species harbors papillomaviruses which are closely related to their human counterparts. J. Virol. **76:** 12537-12542.
3. **Antonsson, A., and N. A. McMillan.** 2006. Papillomavirus in healthy skin of Australian animals. J. Gen. Virol. **87:**3195-200.
4. **Brandsma, J.** 1994. Animal models of human-papillomavirus-associated oncogenesis. Intervirology **37:**189-200.
5. **Brodsky, J. L., and J. M. Pipas.** 1998. Polyomavirus T antigens: molecular chaperones for multiprotein complexes. J. Virol. **72:**5329-34.
6. **Cantalupo, P., A. Doering, C. S. Sullivan, A. Pal, K. W. C. Peden, A. M. Lewis, and J. M. Pipas.** 2005. Complete nucleotide sequence of polyomavirus SA12. J. Virol. **79:**13094-13104.
7. **Castresana, J.** 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. **17:**540-52.
8. **Chan, S.-Y., H. Delius, A. L. Halpern, and H. Bernard.** 1995. Analysis of genomic sequences of 95 papillomavirus types: uniting typing, phylogeny, and taxonomy. J. Virol. **69:**3074-3083.
9. **Cole, C. N., and S. D. Conzen.** 2001. *Polyomaviridae*: The viruses and their replication, p. 2141-2174. *In* D. M. Knipe and P. M. Howley (ed.), Field's Virology, 4th ed., vol. 2. Lippincott Williams and Wilkins, Philadelphia.

10. **Crandall, K. A., M. Perez-Losada, R. G. Christensen, D. A. McClellan, and R. P. Viscidi.** 2005. Phylogenomics and molecular evolution of polyomaviruses, Chapter 2. *In* N. Ahsan (ed.), Polyomaviruses and Human Diseases, vol. 577. Springer, New York.

11. **de Villiers, E., C. Fauquet, T. R. Broker, H. Bernard, and H. zur Hausen.** 2004. Classification of papillomaviruses. Virology **324:**17-27.

12. **DeCaprio, J. A., J. W. Ludlow, and J. Figge.** 1988. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell **54:**275-283.

13. **Delius, H., M. Van Ranst, A. B. Jenson, H. zur Hausen, and J. P. Sundberg.** 1994. Canine oral papillomavirus genomic sequence: a unique 1.5-kb intervening sequence between the E2 and L2 open reading frames. Virology **204:**447-452.

14. **Forslund, O., A. Antonsson, P. Nordin, B. Stenquist, and B. G. Hansson.** 1999. A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. J. Gen. Virol. **80:**2437-2443.

15. **Gaynor, A. M., D. M. Nissen, D. M. Whiley, I. M. Mackay, P. F. Lambert, W. Guang, D. C. Brennan, G. A. Storch, T. P. Sloots, and D. Wang.** 2007. Identification of a novel polyomavirus from patients with acute respiratory tract infections. PLoS Pathog. **3:**1-10.

16. **Gilbert, M. T. P., T. Haselkorn, M. Bunce, J. J. Sanchez, S. B. Lucas, L. D. Jewell, E. Van Marck, and M. Worobey.** 2007. The isolation of nucleic acids from fixed, paraffin-embedded tissues–which methods are useful when? PLoS ONE **June:**e537.

17. **Graffi, A., T. Schramm, I. Graffi, D. Bierwolf, and E. Bender.** 1968. Virus associated skin tumors of the Syrian hamster: preliminary note. J. Natl. Cancer Inst. **40:**867-873.

18. **Gravitt, P. E., C. L. Peyton, T. Q. Alessi, C. M. Wheeler, A. Coutlee, A. Hildesheim, M. H. Schiffman, D. R. Scott, and R. J. Apple.** 2000. Improved Amplification of Genital Papillomaviruses. J. Clin. Microbiol. **38:**357-361.

19. **Groff, D. E., and W. D. Lancaster.** 1985. Molecular cloning and nucleotide sequence of deer papillomavirus. J. Virol. **56:**85–91.

20. **Guerin, J., J. Gelfi, L. Dubois, A. Vuillaume, C. Boucraut-Baralon, and J. Pingret.** 2000. A novel polyomavirus (Goose Hemorrhagic Polyomavirus) is the agent of hemorrhagic nephritis enteritis of geese. J. Virol. **74:**4523-4529.

21. **Hall, K. T., M. E. B. Zajdel, and G. E. Blair.** 1999. Analysis of DNA virus proteins involved in neoplastic transformation, p. 209-245. *In* A. J. Cann (ed.), DNA Viruses: A practical approach. Oxford University Press, Oxford, UK.

22. **Howley, P. M., and D. R. Lowy.** 2001. Papillomaviruses and their replication, p. 2197-2230. *In* D. M. Knipe and P. M. Howley (ed.), Field's Virology, 4th ed., vol. 2. Lippincott Williams and Wilkins, Philadelphia.

23. **Jenson, A. B., J. D. Rosenthal, C. Olsen, F. Pass, W. D. Lancaster, and K. Shah.** 1980. Immunologic relatedness of papillomaviruses from different species. J. Natl. Cancer Inst. **64:**495-500.

24. **Johne, R., D. Enderlein, H. Nieper, and H. Muller.** 2005. Novel Polyomavirus Detected in the Feces of a Chimpanzee by Nested Broad-Spectrum PCR. J. Virol.**79:**3883-3887.

25. **Johne, R., W. Wittig, D. Fernandez-de-Luco, U. Hofle, and H. Muller.** 2006. Characterisation of Two Novel Polyomaviruses of Birds by Using Multiply Primed Rolling-Circle Amplification of Their Genomes. J. Virol. **80:**3523-3531.

26. **Kumar, S., K. Tamura, and M. Nei.** 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief. Bioinform. **5:**150-163.

27. **Lowy, R. L., and P. M. Howley.** 2001. Papillomaviruses, p. 2231-2265. *In* D. M. Knipe and P. M. Howley (ed.), Field's Virology, 4th ed., vol. 2. Lippincott Williams and Wilkins, Philadelphia.

28. **Luo, D., H. Muller, X. B. Tang, and G. Hobom.** 1994. Expression and DNA binding of budgerigar fledgling disease virus large T antigen J. Gen. Virol. **75:**1267-1280.

29. **Major, E. O.** 2001. Human Polyomavirus, p. 2175-2196. *In* D. M. Knipe and P. M. Howley (ed.), Field's Virology, 4th ed., vol. 2. Lippincott Williams and Wilkins, Philadelphia.

30. **Martens, A., A. De Moor, and R. Ducatelle.** 2001. PCR Detection of Bovine Papilloma Virus DNA in Superficial Swabs and Scrapings from Equine Sarcoids. Vet. J. **161:**280-286.

31. **Muller, H., and R. Nitschke.** 1986. A polyoma-like virus associated with acute disease of fledgling budgerigars (*Melopsittacus undulatus*). Med. Microbiol. Immunol. **175:**1-13.

32. **Narechania, A., Z. Chen, R. DeSalle, and R. D. Burk.** 2005. Phylogenetic incongruence among oncogenic genital alpha human papillomaviruses. J. Virol. **79:**15503-10.

33. **Nicholas, K. B., H. B. Nicholas, and D. W. Deerfield.** 1997. GeneDoc: Analysis and visualization of genetic variation, EMBnet News, vol. 4.

34. **Perez-Losada, M., R. G. Christensen, D. A. McClellan, B. J. Adams, R. P. Viscidi, J. C. Demma, and K. A. Crandall.** 2006. Comparing phylogenetic codivergence between polyomaviruses and their hosts. J. Virol. **80:**5663-5669.

35. **Perrott, M. R. F., J. Meers, G. E. Greening, S. E. Farmer, I. W. Lugton, and C. R. Wilks.** 2000. A new papillomavirus of possums (*Trichosurus vulpecula)* associated with typical wart-like lesions. Arch. Virol. **145:**1247-1255.

36. **Pipas, J. M.** 1992. Common and unique features of T antigens encoded by the polyomavirus group. J. Virol. **66:**3979-3985.

37. **Rector, A., K. Doorslaer, M. Bertelsen, I. K. Barker, R. Olberg, P. Lemey, J. P. Sundberg, and M. Van Ranst.** 2005. Isolation and cloning of the raccoon (*Procyon lotor*) papillomavirus type 1 by using degenerate papillomavirus-specific primers. J. Gen. Virol. **86:**2029-2033.

38. **Rector, A., P. Lemey, R. Tachezy, S. Mostmans, S. Ghim, K. Van Doorslaer, M. Roelke, M. Bush, R. J. Montali, J. O. Joslin, R. D. Burk, A. B. Jenson, J. P. Sundberg, B. Shapiro, and M. Van Ranst.** 2007. Ancient papillomavirus-host co-speciation in Felidae. Genome Biol. **8:**R57.

39. **Rector, A., R. Tachezy, and M. Van Ranst.** 2004. A sequence-independent strategy for detection and cloning of circular DNA virus genomes by using multiply primed rolling-circle amplification. J. Virol. **78:**4993-4998.

40. **Richards, J. D.** 2003. Report on threatened Shark Bay marsupials, western barred bandicoot *Permaeles bougainville*, burrowing bettong *Bettongia lesueur lesueur*, banded hare-wallaby *Lagostrophus facsiatus fasciatus*, and rufous hare-wallabies *Lagostrophus bernieri* and *Lagorchestes hirsutus dorreae*, within the Shark Bay region. CSIRO.

41. **Sambrook, J.** 1978. The molecular biology of the papovaviruses., p. 589-672. *In* D. P. Nayak (ed.), The Molecular Biology of Animal Viruses, 2nd ed. Marcel Dekker, New York.

42. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74:**5463–5467.

43. **Scherneck, S., R. Ulrich, and F. Feunteun.** 2001. The Hamster Polyomavirus - A Brief Review of Recent Knowledge. Viral Genes **21:**93-101.

44. **Seebeck, J. H., P. R. Brown, R. L. Wallis, and C. M. Kemper.** 1990. Bandicoots and Bilbies. Surrey, Beatty & Sons Pty Ltd Australia, Chipping Norton, NSW.

45. **Short, J., B. Turner, C. Majors, and J. Leone.** 1997. The fluctuating abundance of endangered mammals on Bernier and Dorre Islands, Western Australia - conservation implications. Aust. Mammal. **20:**53-61.

46. **Stanley, M. A., P. J. Masterson, and P. K. Nicholls.** 1997. *In vitro* and animal models for antiviral therapy in papillomavirus infections. Antivir. Chem. Chemother. **8:**381-400.

47. **Sundberg, J. P., M. K. O'Banion, A. Shima, C. Knupp, and M. E. Reichmann.** 1998. Papillomas and carcinomas associated with a papillomavirus in European harvest mice (*Micromys minutus*). Vet. Pathol. **25:**356-361.

41. **Sundberg, J. P., M. Van Ranst, R. D. Burk, and A. B. Jenson.** 1997. The nonhuman (animal) papillomaviruses: host range, epitope conservation, and molecular diversity. *In* G.

Gross and G. von Krogh (ed.), Human papillomavirus infections in dermatovenereology. CRC Press, Boca Raton.

49. **Tachezy, R., G. Duson, A. Rector, A. B. Jenson, J. P. Sundberg, and M. Van Ranst.** 2002a. Cloning and Genomic Characterisation of *Felis domesticus* Papillomavirus Type 1. Virology **301:**313-321.

50. **Terai, M., and R. D. Burk.** 2002. Felis domesticus papillomavirus, isolated from a skin lesion, is related to canine oral papillomavirus and contains a 1·3 kb non-coding region between the E2 and L2 open reading frames. J. Gen. Virol. **83:**2303-2307.

51. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673-4680.

52. **Tjian, R.** 1978. The binding site on SV40 DNA for a T antigen related protein. Cell **13:**165-179.

53. **Van Ranst, M., J. B. Kaplan, J. P. Sundberg, and R. D. Burk.** 1995. Molecular evolution of papillomaviruses. *In* A. Gibbs, C. H. Calister, and F. Garcia-Arenal (ed.), Molecular basis of virus evolution. Cambridge University Press, Cambridge.

54. **Varsani, A., E. van der Walt, L. Heath, E. P. Rybicki, A. L. Williamson, and D. P. Martin.** 2006. Evidence of ancient papillomavirus recombination. J. Gen. Virol. **87:**2527-31.

55. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1:**945-951.

56. **Woolford, L., A. J. O'Hara, M. D. Bennett, M. Slaven, R. Swan, F. J.A., A. Ducki, C. Sims, S. Hill, P. K. Nicholls, and K. S. Warren.** 2007. Cutaneous papillomatosis and carcinomatosis in the western barred bandicoot (*Perameles bougainville*). Vet. Pathol., in press.

57. **Xia, X., and X. Xie.** 2001. DAMBE: software package for data analysis in molecular biology. J. Hered. **92:**371-373.

58. **zur Hausen, H.** 1996. Papillomavirus infections--a major cause of human cancers. Biochim. Biophys. Acta **1288:**55-78.44.