JOURNAL OF VIROLOGY, Dec. 2007, p. 13280–13290 Vol. 81, No. 24
0022-538X/07/$08.00+0 doi:10.1128/JVI.01662-07

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*▽

Lucy Woolford,[1]* Annabel Rector,[2] Marc Van Ranst,[2] Andrea Ducki,[1] Mark D. Bennett,[1] Philip K. Nicholls,[1] Kristin S. Warren,[1] Ralph A. Swan,[1] Graham E. Wilcox,[1] and Amanda J. O'Hara[1]

School of Veterinary and Biomedical Sciences, Murdoch University, Perth, Western Australia,[1] and Laboratory of Clinical and Epidemiological Virology, Department of Microbiology and Immunology, Rega Institute for Medical Research, University of Leuven, Leuven, Belgium[2]

Received 31 July 2007/Accepted 13 September 2007

Conservation efforts to prevent the extinction of the endangered western barred bandicoot (*Perameles bougainville*) are currently hindered by a progressively debilitating cutaneous and mucocutaneous papillomatosis and carcinomatosis syndrome observed in captive and wild populations. In this study, we detected a novel virus, designated the bandicoot papillomatosis carcinomatosis virus type 1 (BPCV1), in lesional tissue from affected western barred bandicoots using multiply primed rolling-circle amplification and PCR with the cutaneotropic papillomavirus primer pairs FAP59/FAP64 and AR-L1F8/AR-L1R9. Sequencing of the BPCV1 genome revealed a novel prototype virus exhibiting genomic properties of both the *Papillomaviridae* and the *Polyomaviridae*. Papillomaviral properties included a large genome size (~7.3 kb) and the presence of open reading frames (ORFs) encoding canonical L1 and L2 structural proteins. The genomic organization in which structural and nonstructural proteins were encoded on different strands of the double-stranded genome and the presence of ORFs encoding the nonstructural proteins large T and small t antigens were, on the other hand, typical polyomaviral features. BPCV1 may represent the first member of a novel virus family, descended from a common ancestor of the papillomaviruses 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.

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 to exist only on Bernier and Dorre Islands in the World Heritage Area of Shark Bay, Western Australia (41, 45, 46). Conservation efforts to prevent the extinction of the WBB are currently hampered by a progressively debilitating cutaneous and mucocutaneous 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 mucocutaneous junctions of the lips and conjunctiva. Histological, ultrastructural, and immunohistochemical features of the lesions support the involvement of a papillomavirus (PV) or polyomavirus (PyV) in the pathogenesis of this disease (57).

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 (28, 49). These viruses exhibit a range of pathogenicity and have been associated with both benign and malignant disease (4, 47, 48, 59). Like PVs, PyVs infect humans and a variety of mammalian and avian species, with 14 PyV types completely genomically characterized to date (15, 25, 35). 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 into an unnatural host (9, 30, 42). The hamster PyV (HaPyV) has the ability to naturally infect hair follicle keratinocytes and induce cutaneous epitheliomas (17, 44). 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, 32).

PVs and PyVs were previously considered subfamilies of *Papovaviridae*. Both are small nonenveloped 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 organizations and

* Corresponding author. Mailing address: School of Veterinary and Biomedical Sciences, Murdoch University, South Street, Murdoch, Western Australia 6150. Phone: 618 9360 2479. Fax: 618 9310 4144. E-mail: l.woolford@murdoch.edu.au.

▽ Published ahead of print on 26 September 2007.

TABLE 1. WBBs screened for BPCV1 DNA using superficial skin swabs

Origin	No. of WBBs examined and swabbed	No. of WBBs with clinical evidence of skin disease
Dryandra Woodland[a]	18 (6[b])	6 (5[b])
Bernier Island[c]	24	2
Dorre Island[c]	20	0
Heirisson Prong Biosphere[d]	11	0
Total	73	8

[a] Captive breeding facility, Narrogin, Western Australia.
[b] Currently quarantined at Kanyana Wildlife Rehabilitation Centre or deceased.
[c] Remnant wild population, Shark Bay, Western Australia.
[d] Extensive-range captive breeding facility, SharkBay, Western Australia.

TABLE 2. WBB origins and histological classification of biopsied lesions from which BPCV1 DNA was amplified by RCA

WBB	Origin	Biopsy anatomical site and histological classification
1	KWRC[a]	Skin from digit, carcinoma in situ
2	Dryandra Woodland	Lip mucocutaneous junction, papillomatous hyperplasia
3	Dryandra Woodland	Right eyelid, carcinoma in situ Skin lesion from the ventral tail, papillomatous hyperplasia
4	PCBC[b]	Left eyelid, papillomatous hyperplasia

[a] KWRC, Kanyana Wildlife Rehabilitation Centre.
[b] PCBC, Peron Captive Breeding Centre.

encode different numbers and types of structural and nonstructural proteins, they are currently recognized as two separate virus families by the International Committee on the Taxonomy of Viruses (13, 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 skins of Australian marsupial species, namely, from a cutaneous papilloma of a common brush-tail possum (*Trichosurus vulpecula*) (36) and from the healthy skin of koalas (*Phascolarctos cinereus*) and an eastern gray kangaroo (*Macropus giganteus*) (3). However, complete genomic sequences were not deduced in these studies; rather, sequencing was restricted to 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 bandicoot papillomatosis carcinomatosis virus type 1 (BPCV1). BPCV1 was amplified from lesional tissue and from skin swabs taken from the surfaces of lesions from WBBs affected by a papillomatosis and carcinomatosis syndrome.

MATERIALS AND METHODS

Clinical material. Between 2000 and 2007, cutaneous and mucocutaneous lesional and nonlesional tissue samples from 32 WBBs affected by a papillomatosis and carcinomatosis syndrome (57) 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 in 10% neutral buffered formalin for histological evaluation. Submissions from the remaining 13 individuals were collected at a referring wildlife breeding center 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 (57). All submissions were received from captive populations of WBBs held at either Kanyana Wildlife Rehabilitation Center, Dryandra Woodland captive breeding facility, or the Peron Captive Breeding Center in Western Australia.

Collection of superficial skin swabs. Superficial skin swabs collected for the purpose of viral-DNA detection were taken from 73 WBBs examined during field monitoring activities carried out between 2005 and 2007 using sterile 0.9% NaCl-soaked cotton-tipped swabs following the methods of Martens et al. and Antonsson et al. (2, 31). The number and origin of WBBs examined during this study and the number of individuals with clinical evidence of the papillomatosis and carcinomatosis syndrome are presented in Table 1. Sites swabbed for each individual included cutaneous or mucocutaneous lesions if present; the skin of the lateral flank, lip commissure, and feet; and the conjunctival epithelium. Swabs were rubbed against the skin or lesion surface 6 to 10 times and then placed into sterile saline within a 1.5-ml microcentrifuge tube and stored at −20°C. In the laboratory, microcentrifuge tubes containing the cotton-tipped swabs were thawed and vortexed for 30 seconds. Each sample was spun down briefly, following which the cotton-tipped swabs were removed and discarded using sterile forceps and the remaining suspension was stored at −20°C until further testing was done.

DNA extraction from skin lesions. Sixty-three fresh tissue 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 tissue.

Multiply primed RCA. Multiply primed rolling-circle amplification (RCA) was performed on 13 lesional extracts from six affected WBBs using the TempliPhiTM 100 Amplification Kit (Amersham Biosciences) and a modified protocol optimized for the amplification of viral circular DNA genomes (40). One microliter of extracted WBB lesional DNA (containing 1 to 5 μg of total DNA) and 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 min, and then placed on ice. For each sample, a premixture was prepared on ice by mixing 5 μl TempliPhi Reaction Buffer, 0.2 μl TempliPhi enzyme mix containing the ϕ29 polymerase, and an extra 450 μM of each deoxynucleoside triphosphate (dNTP). After being vortexed, 5 μl of the premixture was added to each of the cooled samples, and the reaction mixtures were gently mixed and incubated overnight at 30°C. Following overnight incubation, the reaction mixtures were heated to 65°C for 10 min 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 U of BamHI or SalI (Promega) 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 by ethidium bromide staining to check for the presence of a DNA band consistent with full-length PV 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 to 4 (Table 2) with either BamHI or SalI resulted in one DNA fragment of approximately 7.5 kb. RCA products obtained from the lip lesion of WBB 2 and the eyelid lesion of WBB 3 were selected for cloning. A 5-μl aliquot of each RCA product was digested with 40 U of BamHI or SalI, 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 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 (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 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 BamHI-cut ~7.5-kb DNA fragment or the SalI-cut ~7.5-kb DNA fragment obtained by digestion of RCA

products from the lip lesion of WBB 2 and the eyelid lesion of WBB 3, respectively, were selected for sequencing. The complete genome of BPCV1 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 its entirety. Sequencing of the purified plasmids was carried out using the dideoxynucleotide chain termination method (43). The sequence was determined using an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA). 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 (http://www.mbio.ncsu.edu/BioEdit/).

Long-template PCR. Long-range PCR primers, designed from BPCV1 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 BPCV1 genome independently of RCA. Primers were chosen to produce amplicons encompassing the regions linking the PV-like and PyV-like open reading frames (ORFs) of the BPCV1 genome in order to confirm the occurrence of both PV- and PyV-like ORFs in a single viral genome. The 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 2,130 nucleotides (nt) linking the large T and L1 ORFs of BPCV1, and LTPCR-L2F2 in L2 (5′-CACCACCTCTACCTGTTCCTATACCTAATCCACC-3′) and LTPCR-STR2 in small t (5′-CATCACCTTCACTCTCGTAGCAGAACAGATCTTCC-3′) for a second fragment of 1,259 nt 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 min of denaturation at 94°C, followed by 10 cycles of 10 seconds of denaturation at 94°C, 30 seconds of annealing at 63°C, and 4 min of elongation at 68°C. This was followed by 25 cycles of 10 seconds of denaturation at 94°C, 30 seconds of annealing at 63°C, and 4 min of elongation at 68°C, with an increase in elongation time by 20 additional seconds each cycle. After a final elongation step of 7 min at 68°C, the samples were 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 1,531-nt stretch of the first amplicon, and with primers L2F2 (5′-CTGTTCCTATACCTAATCCACC-3′) and STR2 (5′-GCCATCACAAGTATCACAACC-3′), 1,051 nt of the second amplicon was sequenced.

PCR detection using degenerate PV-specific primers. PCR with degenerate cutaneotropic PV-specific primers was performed on RCA products, tissue DNA extracts, and skin swab suspensions with the degenerate primer pair AR-L1F8/AR-L1R9 (38) 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 primers, 1 U of *Taq* DNA polymerase, 1.5 mM $MgCl_2$ (pH 8.5), and 1× DNA polymerase reaction buffer, with 2 μl of 1:10-diluted RCA product, extracted lesional DNA, or skin swab suspension as a template. PCR reagents were supplied by Fisher Biotech Australia or Perkin Elmer/Roche Molecular Systems. PCR was carried out in an automated thermocycler (Gene Amp PCR System 2400; Perkin Elmer, Foster City, CA) programmed for block temperatures, using the following parameters: 10 min at 94°C and then 45 cycles of 1.5 min at 94°C, 1.5 min at 50°C, and 1.5 min at 72°C, followed by 5 min 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 used for PCR. The sequences of the PCR products were 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 BPCV1-specific primers. PCR with BPCV1-specific primers was performed on RCA products, tissue DNA extracts, and skin swab suspensions with the primer pair LWL1F (5′-GAGGAGGGACATCAGGTGAC-3′)/LWL1R (5′-ATTGTTTTGCCCAGTTGCTC-3′) designed within the BPCV1 L1 ORF to produce a 176-bp 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 primers, 0.5 U of *Taq* DNA polymerase, 1.5 mM $MgCl_2$ (pH 8.5), and 1× DNA polymerase reaction buffer, with 2 μl of 1:10-diluted RCA product, extracted lesional DNA, or skin swab suspension as a template. PCR reagents were supplied by Fisher Biotech Australia, Perth, Western Australia. PCR was carried out in an automated thermocycler (Gene Amp PCR System 2400; Perkin Elmer, Foster City, CA) programmed for block temperatures, using the following parameters: 5 min 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 min at 72°C. The PCR products were purified by 2% agarose gel electrophoresis, extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen), and sequenced with the same primers used for PCR. The sequences of the PCR products were determined and edited as described above for plasmids.

DNA and protein sequence analysis. The putative 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 server (version 2.2.13) on the NIH genetic sequence database GenBank (1). Protein sequence similarities between BPCV1 and known PVs and PyVs 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 BPCV1 sequence to other PV types, phylogenetic analysis was performed on the nucleotide sequences of the L1 and L2 ORFs of BPCV1, 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 (52) using the BLOSUM protein weight matrix in the DAMBE software package version 4.2.13 (58). This amino acid alignment was then used as a template for aligning the corresponding nucleotide sequences in DAMBE. The nucleotide alignments were corrected manually in the GeneDoc Multiple Sequence Alignment Editor and Shading Utility software package version 2.6.002 (34), 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 (27).

For the BPCV1 large T antigen, 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 BPCV1 large T antigen gene could not be confidently identified, the analysis was performed on the complete large T antigen genomic sequences, thus including intron sequences. The large T antigen nucleotide sequences were aligned with the ClustalW algorithm (52) implemented in the DAMBE software package version 4.2.13 (58). 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.

Nucleotide sequence accession number. The nucleotide sequence data reported in this article were deposited in GenBank using the NCBI (Bethesda, MD) BankIt v3.0 submission tool (http://www.ncbi.nlm.nih.gov/BankIt/) under accession number EU069819.

RESULTS

Detection of a novel virus using multiply primed RCA, degenerate-primer PCR, and long-template PCR. BamHI or SalI digestion of RCA products amplified from five lesional biopsy specimens collected from WBBs 1 to 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 that the complete genome of a novel virus had been amplified. A 450-bp DNA fragment was amplified with the FAP59/FAP64 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 PV 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

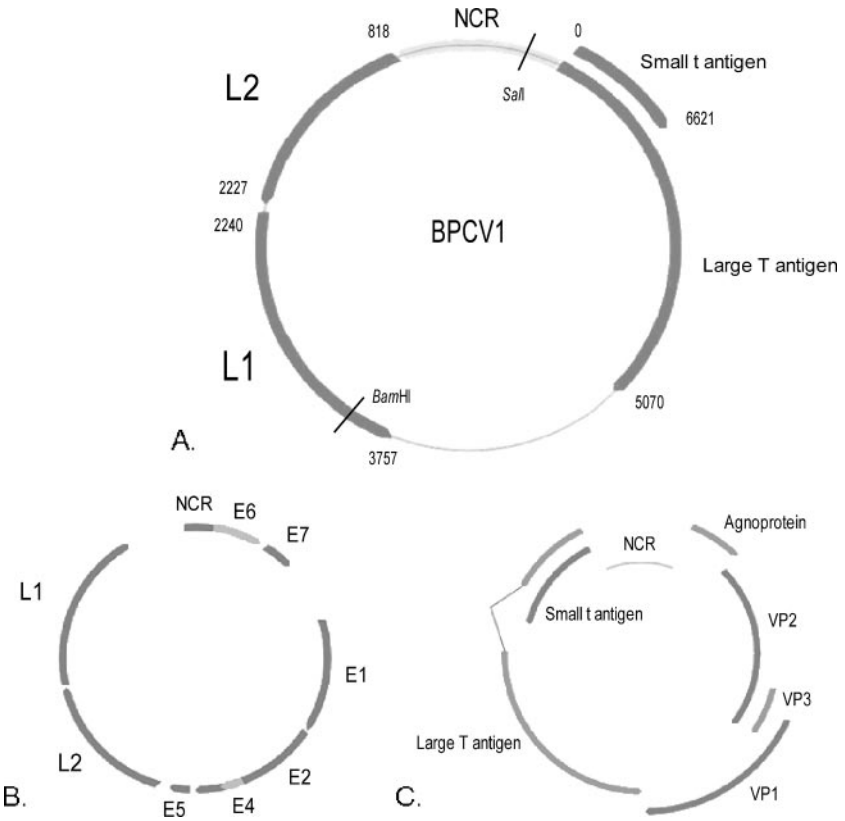

FIG. 1. Genomic organization of BPCV1 (A) and schematic representation of classical *Papillomaviridae* (HPV16) (B) and *Polyomaviridae* (JCV) (C) genomic organizations. BPCV1 ORFs are divided between both strands of the genome, with the structural proteins encoded on one strand and the nonstructural proteins encoded on the other strand. The numbers show the nucleotide positions of the putative ORF start and stop codons. BamHI and SalI cut sites are indicated.

of RCA. Amplicons were generated that were identical to BPCV1 sequences obtained by sequencing of cloned RCA products and that encompassed sequences from both the early and late ORFs, revealing that early- and late-region ORFs were continuous with one another on template DNA (BPCV1 genome) and that the sequences were identical to those retrieved from the cloned RCA products.

Analysis of BPCV1 complete genomic sequence and deduced amino acid sequences. The newly identified virus was tentatively named BPCV1, and its complete genome was 7,295 bp, with a GC content of 37.4%. The genomes of the novel virus amplified from the lip lesion of WBB 2 and an eyelid lesion of WBB 3 were sequenced in their entirety and found to be identical. ORF analysis and BLASTX searches predicted the presence of the two classical PV late (L) protein-like ORFs and two PyV T-antigen-like early-region ORFs. Putative T antigens were encoded on the opposing strand to the late-protein ORFs (Fig. 1A).

The genome of BPCV1 exhibited two ORFs encoding proteins demonstrating the greatest similarity to the PV major (L1) and minor (L2) capsid proteins. The putative L1 ORF was 1,518 bp in length (nt 2240 to 3757), and the putative L2 was 1,409 bp (nt 818 to 2227) in length. The sequence similarities between the L1 and L2 ORFs of BPCV1 and human papillomavirus type 1 (HPV1) (a benign cutaneous PV of the genus *Mupapillomavirus*; NC_001356), HPV5 (an epidermodysplasia verruciformis-associated PV of the genus *Betapapillomavirus*; NC_001531), HPV16 (a mucosal high-risk PV of the genus *Alphapapillomavirus*; NC_001526), HPV6 (a mucosal low-risk PV of the genus *Alphapapillomavirus*; X00203), and bovine BPV1 (a fibropapillomavirus of the genus *Deltapapillomavirus*; X02346) were investigated by pairwise alignments performed at the amino acid level. The similarities of the putative BPCV1 L1 and L2 sequences to those of the PV types listed above ranged between 47 and 51% and 26 and 33%, respectively (Table 3). The BPCV1 late ORFs did not demonstrate any similarity to classical PyV capsid protein-encoding ORF VP1, VP2, or VP3.

In addition to the late proteins described above, two BPCV1 ORFs coded for proteins demonstrating the greatest similarity to the avian and mammalian PyV T antigens: a putative large T antigen (nt 7295 to 5070) and a putative small t antigen (nt

TABLE 3. Percentages of amino acid similarity of the BPCV1 L1 and L2 ORFs with those of HPV1, HPV5, HPV16, HPV6, and BPV1

BPCV1 ORF	% Amino acid similarity to ORF of:				
	HPV1	HPV5	HPV16	HPV6	BPV1
L1	51	55	48	50	47
L2	29	33	30	29	26

TABLE 4. Percentages of amino acid similarity of the BPCV1 large T antigen and small t antigen ORFs with those of known PyVs

BPCV1 ORF	Predicted size (aa)	% Amino acid similarity with ORF of:												
		Avian				Eutherian								
		CyPV	FPyV	GHPyV	BFPyV	BPyV	BKV	JCV	SV40	SA12	HaPyV	LPV	PyV	KV
Small t antigen	224	20	16	22	16	6	5	6	6	7	5	4	5	0
Large T antigen	742	35	33	32	32	25	23	24	23	24	27	27	19	24

7295 to 6621). The sequence similarities between the respective large T antigen and small t antigen ORFs of BPCV1 and 13 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 MPyV [MptV] NC_001505, bovine PyV [BPyV] NC_001442, JC virus [JCV] NC_001699, BK virus [BKV] NC_001538, budgerigar fledgling disease virus [BFPyV] NC_004764, goose hemorrhagic disease PyV [GHPyV] NC_004800, crow PyV [CPyV] NC_007922, and finch PyV [FPyV] NC_007923) were 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 BPCV1 large T antigen ORF. Therefore, alignments were performed using the BPCV1 large T protein sequence predicted from the unspliced ORF nucleotide sequence. The similarities of the putative BPCV1 large T antigen and small t antigen sequences to those of known PyVs ranged between 19 and 35% and 0 and 22%, respectively (Table 4).

Further analysis of these proteins revealed conserved PyV T-antigen motifs. A DnaJ domain was located between amino acids (aa) 8 and 72 of BPCV1 large T antigen and small t antigen proteins, including the conserved hexapeptide HP DKGG (Table 5), and an ATPase binding domain (GPVNT GKT) was present between aa 543 and 550. The conserved PyV T antigen LXCXE motif, LFCYE, was found located between aa 78 and 82 within both the putative large T and small t antigens (Table 5). 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-437, and H-441 (Table 5).

The genomic organization of BPCV1 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 to 817). Two GAGGC pentanucleotides were located within this region and may represent potential T-antigen binding sites. A second noncoding region was also found to lie between the end of L1 and the end of the large T antigen ORFs (nt 3758 to 5069). No recognizable regulatory or promoter elements were identified in this region, and BLAST searches performed with the region did not reveal any similarity to known PVs or PyVs.

Phylogenetic analysis. Since BPCV1 shows similarity to PyV genomes in its early region but has a late region encoding PV proteins, the evolutionary history of BPCV1 was reconstructed separately for the two regions, based on comparison to the PyV large T antigen and the PV L1 and L2 capsid genes. A neighbor-joining phylogenetic tree was constructed based on an alignment of the large T antigen sequences of BPCV1 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. BPCV1 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 yet. This corroborates a scenario in which the BPCV1 early region would have branched off from the PyV evolutionary tree very early in evolution.

An L1 neighbor-joining phylogenetic tree was constructed based on alignment of the L1 ORF of BPCV1 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 genus *Betapapillomavirus*, 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 PVs, BPCV1 did not share a common branch with the members of the genus *Betapapillomavirus*. Instead, it originated near the root of the evolutionary tree in a common branch with the *Gammapapillomavirus* and the rodent PVs hamster oral PV (HaOPV), *Mastomys coucha* PV (McPV), and *Micromys minutus* PV (MmPV) (data not shown). Again, this clustering showed only low bootstrap support.

Screening of WBB papillomas, carcinomas, and nonlesional epithelia by PCR. BPCV1 was amplified from the skin lesions of 18/19 (94.7%) affected WBBs from which DNA was extracted from fresh lesional tissues. In affected WBBs for which only formalin-fixed tissues were available, BPCV1 was detected in skin lesions from 4/13 (30.7%). Histological classification and PCR results for lesions screened for BPCV1 DNA are presented in Tables 6 and 7. Both FAP59/FAP64 and BPCV1-specific primer pairs were utilized in tissue screening, and sequencing of amplicons indicated that only viral sequences identical to BPCV1 were amplified from these tissue extracts. No viral DNA was amplified from cutaneous and mucosal tissue extracts from the two unaffected WBBs.

Screening of skin swabs taken from WBBs in the field. Superficial skin swabs taken from the surfaces of lesions from each of the eight WBBs found to be clinically affected by the papillomatosis and carcinomatosis syndrome tested positive for BPCV1 DNA. Both FAP59/FAP64 and BPCV1-specific primer pairs were utilized, and sequencing of amplicons indicated that only viral sequences identical to BPCV1 were amplified from skin swab suspensions. Affected WBBs that tested

TABLE 5. Conserved PyV T-antigen motifs encoded by BPCV1 early domain

Virus	GenBank accession no.	Sequence[b]
HPDKGG box,[a] large T antigen		
BPCV1	EU069819	^{34}KM**HPDKGG**STNAMKRLNELNQ^{54}
CPyV	NC_007922	^{37}MY**HPDKGG**DEEKMKRLNQLMD^{57}
FPyV	NC_007923	^{32}EF**HPDKGG**DPERMKELNRLMD^{52}
GHPyV	NC_004800	^{34}MF**HPDKGG**DEEKMKRLNYLME^{54}
BFPyV	NC_004764	^{32}KY**HPDKGG**DEEKMKELNTLME^{52}
BKV	NC_001538	^{40}EF**HPDKGG**DEDKMKRMNTLYK^{60}
JCV	NC_001699	^{40}EL**HPDKGG**DEDKMKRMNFLYK^{60}
SV40	NC_001669	^{40}EF**HPDKGG**DEEKMKKMNTLYK^{60}
SA12	NC_007611	^{40}EF**HPDKGG**DEDKMKRMNTLYK^{60}
LPyV	NC_004763	^{40}LY**HPDKGG**DSAKMQRLNELFQ^{60}
HaPV	NC_001663	^{40}QL**HPDKGG**NEELMQQLNTLWT^{60}
PyV	NC_001515	^{40}LL**HPDKGG**SHALMQELNSLWG^{60}
MptV	NC_001505	^{40}IV**HPDKGG**SDELSQELISLYR^{60}
Cr2 LXCXE Rb binding area		
BPCV1	EU069819	^{75}ED**LFCYE**SEGDDEPDTC^{91}
CPyV	NC_007922	^{67}ET**LQCEE**TLYSSDEEDV^{83}
FPyV	NC_007923	^{58}QS**LFCDE**TLDSDSDDGD^{74}
GHPyV	NC_004800	^{63}ED**LFCDE**ELSSSEEEDV^{79}
BFPyV	NC_004764	^{58}EG**LRADE**TLEDSDPEPE^{74}
BPyV	NC_001442	^{58}QD**LHCDE**ELEPSDNEEE^{74}
BKV	NC_001538	^{103}ED**LFCHE**DMFASDEEAT^{119}
JCV	NC_001699	^{103}ED**LFCHE**EMFASDDENT^{119}
SV40	NC_001669	^{101}EN**LFCSE**EMPSSDDEAT^{117}
SA12	NC_007611	^{103}ED**LFCHE**DMFQSDEEGT^{119}
LPyV	NC_004763	^{129}DD**LFCSE**TMSSSSDEDT^{145}
HaPV	NC_001663	^{128}ED**LTCQE**ELSSSEDEFT^{144}
PyV	NC_001515	^{140}PD**LFCYE**EPLLSPNPSS^{156}
MptV	NC_001505	^{101}FD**LFCNE**AFDRSDDEQE^{117}
Zinc finger motifs		
BPCV1	EU069819	^{411}NTIQG**CKLC**VKDQLLLTGLHKNHAKD--**HVQHH**ENA^{444}
CPyV	NC_007922	^{293}VNPDE**CKDC**KEDRENSSLQRLKRRRPGG**HLEDH**ATH^{328}
FPyV	NC_007923	^{275}RPVEA**CPDC**AKERETAKRQRTSHLED--**HPA-H**QKN^{307}
GHPyV	NC_004800	^{289}VPQEE**CADC**QSQKENLCFGQLKRKWYGG**HLDDH**GIH^{324}
BFPyV	NC_004764	^{253}QPTDK**CPEC**QKDKDTVKRKRSTHIDD--**HPR-H**QHN^{285}
BPyV	NC_001442	^{254}TAPEA**CKVC**DNPRRLEHRRH————————**HTKDH**TLN^{281}
BKV	NC_001538	^{299}YNVEE**CKKC**QKKDQPYHFKY————————**HEK-H**FAN^{325}
JCV	NC_001699	^{298}ENPQQ**CKKC**EKKDQPNHFNH————————**HEK-H**YYN^{324}
SV40	NC_001669	^{297}YSFEM**CLKC**IKKEQPSHYKY————————**HEK-H**YAN^{323}
SA12	NC_007611	^{298}HNPEE**CRKC**QKKEQPYHFKF————————**HEK-H**FAN^{325}
LPyV	NC_004763	^{362}VEPGK**CGKC**EKKQHKFHYNY————————**HKA-H**HAN^{388}
HaPV	NC_001663	^{421}QCESS**CKKC**AEALPRMKVHWAN————————**HSQ-H**LEN^{449}
MPyV	NC_001515	^{443}KEVPS**CIKC**SKEETRLQIHWKN————————**HRK-H**AEN^{471}
MptV	NC_001505	^{314}SPVPN**CSKC**ENRMLTNHFKF————————**HKE-H**HEN^{340}

[a] HPDKGG box not encoded by BPyV.
[b] Conserved motifs are in boldface.

positive for BPCV1 originated from a wild population on Bernier Island ($n = 2$) and from a captive breeding colony at Dryandra Woodland ($n = 6$). BPCV1 was not detected in skin swab suspensions taken from the surfaces of normal cutaneous and mucosal epithelia in the 65 clinically normal WBBs examined in the field.

DISCUSSION

BPCV1 was found to possess a double-stranded circular DNA genome 7,295 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 nonstructural 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) only one DNA strand (9, 22).

The putative BPCV1 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 (13). BPCV1 L1 shared less than 60% identity with the L1s 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 BPCV1 L1 sequence was sufficiently similar to those of cutaneous PV types to allow detection with degenerate primer pairs designed to detect a broad range of cutaneotropic PV types.

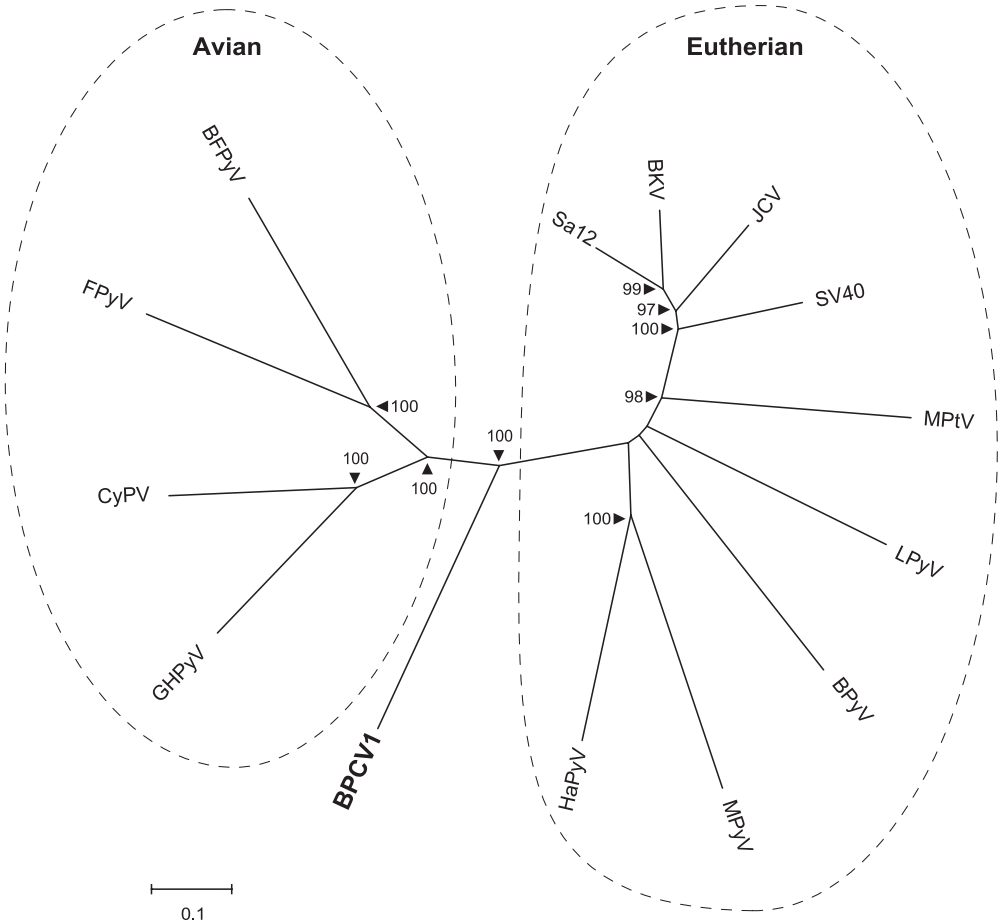

FIG. 2. Large T antigen phylogenetic tree of BPCV1 and 13 established PyVs (LPyV NC_004763, SV40 NC_001669, Sa12 NC_007611, HaPyV NC_001663, MPyV NC_001515, MptV NC_001505, BPyV NC_001442, JCV NC_001699, BKV NC_001538, BFPyV NC_004764, GHPyV NC_004800, CPyV NC_007922, and FPyV NC_007923) showing the evolutionary relationship of the BPCV1 sequence to members of the *Polyomaviridae*. Phylogenetic reconstruction was performed by the neighbor-joining method. Bootstrap percentages (based on 10,000 iterations) of ≥80% are shown at internal nodes (arrowheads), and the genetic distance in nucleotide substitutions per site is indicated by the scale bar. The clusters of PyVs that infect avian and eutherian host species, respectively, are indicated.

Two predicted BPCV1 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 BPCV1 genome to those of known PyVs, it was not possible to predict T-antigen mRNA splice junctions from this sequence analysis alone. Examination of BPCV1 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 BPCV1 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 (37). 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 BPyV large T antigen, all characterized PyV large T antigens and small t antigens contain the conserved HPDKGG motif (37). The PyV ATPase binding domain, GP(V/I)(N/D)XGKT (56), was also identified within BPCV1 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 (37). A large T antigen consensus motif, LXCXE, required for binding to the tumor suppressor protein Rb and to two structurally related proteins,

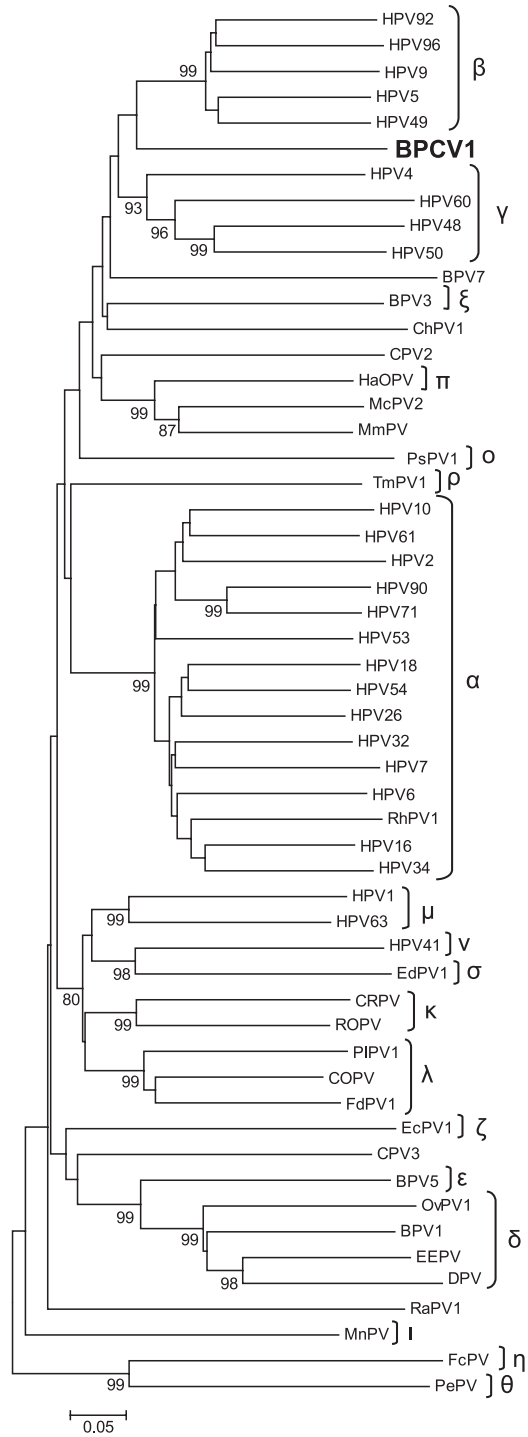

FIG. 3. Evolutionary relationship of the BPCV1 sequence to members of the *Papillomaviridae*. Shown is a neighbor-joining phylogenetic tree of the L1 sequences of BPCV1 and 53 PVs [genus *Alphapapillomavirus*, HPV32 (NC_001586), HPV10 (NC_001576), HPV61 (NC_001694), HPV2 (NC_001352), HPV26 (NC_001583), HPV53 (NC_001593), HPV18 (NC_001357), HPV7 (NC_001595), HPV16 (NC_001526), HPV6 (NC_001355), HPV34 (NC_001587), RhPV1 (NC_001678), HPV54 (NC_001676), HPV90 (NC_004104), and HPV71 (AY330621); genus *Betapapillomavirus*, HPV5 (NC_001531), HPV9 (NC_001596), HPV49 (NC_001591), HPV92 (NC_004500), and HPV96 (NC_005134); genus *Gammapapillomavirus*, HPV4 (NC_001457), HPV48 (NC_001690), HPV50 (NC_001691), and HPV60 (NC_001693); genus *Deltapapillomavirus*, EEPV (NC_001524), DPV (NC_001523), OvPV1 (NC_001789), and BPV1 (NC_001522); genus *Epsilonpapillomavirus*, BPV5 (NC_004195); genus *Zetapapillomavirus*, EcPV1 (NC_003748); genus *Etapapillomavirus*, FcPV (NC_004068); genus *Thetapapillomavirus*, PePV (NC_003973); genus *Iotapapillomavirus*, MnPV (NC_001605); genus *Kappapapillomavirus*, CRPV (NC_001541) and ROPV (NC_002232); genus *Lambdapapillomavirus*, canine oral PV (COPV) (NC_001619), FdPV1 (NC_004765), and *Procyon lotor* PV (NC_007150); genus *Mupapillomavirus*, HPV1 (NC_001356) and HPV63 (NC_001458); genus *Nupapillomavirus*, HPV41 (NC_001354); genus *Xipapillomavirus*, BPV3 (NC_004197); genus *Omicronpapillomavirus*, PsPV1 (NC_003348); genus *Pipapillomavirus*, HaOPV (E15111); genus *Rhopapillomavirus*, TmPV1 (NC_006563); genus *Sigmapapillomavirus*, EdPV1 (NC_006951); and unclassified types RaPV1 (NC_008298), BPV7 (NC_007612), CPV2 (NC_006564), CPV3 (NC_008297), ChPV1 (NC_008032), McPV2 (DQ664501) and MmPV (NC_008582)]. The established PV genera are indicated by their Greek symbols. The scale bar indicates the genetic distance (in nucleotide substitutions per site), and the numbers at the internal nodes represent the bootstrap probabilities in percents as determined for 10,000 iterations by the neighbor-joining method. Only bootstrap values of 80% or more are shown.

p107 and p130 (21), was identified in BPCV1 as LFCYE. BPCV1 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 Rb binding motif, which is a common feature of both PyV large T and PV E7 proteins.

Based on the PyV-like genomic organization of BPCV1, 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 *ori*, the TATA box, T-antigen binding sites, cellular transcription factor binding sites, and a 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 BFPyV and GHPyV, which do not contain any GAGGC pentanucleotides within the regulatory region (6, 37). In mammalian PyVs, the large T antigen binds to this GAGGC pentanucleotide (53). In avian PyVs, however, the binding sequence for the large T antigen has been identified as the palindromic sequence $TCC(A/T)_6GGA$ or similar (25, 29), for which related sequences were not identified in the BPCV1 sequence. Two potential GAGGC large T antigen binding sites were identified in the putative BPCV1 viral regulatory region.

The noncoding 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 BPCV1 viral regulatory region, which was not unexpected, since BPCV1 does not appear to encode any proteins demonstrating similarity to PV E1 or E2.

A second noncoding region was located between the ends of the BPCV1 L1 and large T antigen ORFs; however, no signif-

TABLE 6. Charateristics of fresh tissues from affected WBBs screened for BPCV1 DNA by PCR

PCR result	Histological classification of lesions[a]				Subtotal (lesional tissue)[a]	Nonlesional skin[a]	Total no. of tissues tested
	Papillomatous hyperplasia ± intranuclear inclusions	Carcinoma in situ	Squamous cell carcinoma	Adenocarcinoma			
BPCV1 positive	20 (95.2)	14 (87.5)	12 (85.7)	0 (0.0)	46 (88.5)	2 (28.6)	48
BPCV1 negative	1 (4.8)	2 (12.5)	2 (14.3)	1 (100.0)	6 (11.5)	5 (71.4)	11
Total	21	16	14	1	52	7	59

[a] Number (percent).

icant homology to known PyV or PV types was found in the region, and we were unable to identify regulatory or promoter elements. A second noncoding region located between the end of the early and the beginning of the late protein region has also been characterized in the *Lambdapapillomavirus* types, including the feline PVs, canine oral PV, and *Procyon lotor* PV (12, 38, 39, 50, 51) and in the deer PV (*Deltapapillomavirus*) (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 (39).

Due to the novel nature of this viral genome, concerns were initially raised that there might be a dual PV-PyV infection in WBB lesional tissue or that contamination of WBB DNA extracts with either PV or PyV DNA might have occurred in the laboratory and therefore this unique hybrid-like PV-PyV genome might 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 BPCV1 template DNA. In addition, the partial sequences of BPCV1 generated using long-template PCR independently of RCA were found to be identical to the BPCV1 sequence obtained from cloned RCA products. The findings that the same-size, 7.3-kb genome was detected in biopsy specimens from four different individuals; that the genomes from the two RCA reactions sequenced in their entirety were identical; and that these sequences were unlike previously characterized PVs and PyVs were also supportive of the existence of this novel genome.

BPCV1 was detectable in 94.7% of affected individuals for which fresh lesional tissue extracts were screened, 30.8% of individuals for which formalin-fixed tissue extracts were screened, and 100% of individuals displaying clinical evidence of the papillomatosis and carcinomatosis syndrome screened by a skin-swabbing technique. Amplification of BPCV1 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). This was supported by the failure to amplify WBB genomic sequences from DNA extracted from the formalin-fixed lesions when a PCR technique that amplified a highly conserved cytochrome *b* region of mammalian mitochondrial DNA was used (26) (data not shown). BPCV1 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, but not in the single case of adenocarcinoma. BPCV1 was not detected in tissue samples or skin swabs taken from clinically normal individuals. It appears that BPCV1 is only detectable in individuals displaying skin lesions and is therefore unlikely to be a commensal of normal WBB skin. No viral sequences other than BPCV1 were amplified using broad-spectrum cutaneotropic PV- and BPCV1-specific primer pairs. In addition to the results presented in this study, screening of WBB lesions using MY09/MY11 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 (data not shown). These results are supportive of the hypothesis that BPCV1 is a necessary factor for the 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.

TABLE 7. Characteristics of formalin-fixed tissues from affected WBBs screened for BPCV1 DNA by PCR

PCR result	Histological classification of lesions[a]			Total[a]
	Papillomatous hyperplasia ± intranuclear inclusions	Carcinoma in situ	Squamous cell carcinoma	
BPCV1 positive	2 (25.0)	0 (0.0)	2 (50.0)	4 (30.8)
BPCV1 negative	6 (75.0)	1 (100.0)	2 (50.0)	9 (69.2)
Total	8	1	4	13

[a] Number (percent).

The observed patterns of disease in captive populations are suggestive that disease is possibly transmitted from affected to unaffected WBBs through direct contact, such as when fighting and mating (57). Earlier studies to examine the transmissibility of this disease using an immunodeficient-mouse model were unsuccessful, and no experimental work has yet been performed to ascertain the infectivity of the BPCV1 genome.

BPCV1, detected in cutaneous and mucocutaneous lesions from the endangered WBB, 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 nonstructural proteins and exhibiting the genomic organization of the *Polyomaviridae*. A quandary has been created in regard to the taxonomic placement of this virus based on currently accepted classification systems. Over 100 PV types have been completely characterized in humans alone (13). An additional 40 complete genomes of nonhuman PVs, retrieved from 31 distinct amniote host species (mammals and birds), are currently available in GenBank. The positions, sizes, and functions of many of the ORFs are well conserved among the various PV types that have been sequenced and studied (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 descendants of a common ancestor (9).

BPCV1 may have arisen following a recombination event between existent (but yet undiscovered) or ancestral members of the families *Papillomaviridae* and *Polyomaviridae* in which a PV provided the late-region structural-gene cassette and a PyV delivered the early-region nonstructural-gene cassette to the recombinant virus. However, although recombination events have been suspected in PVs (33, 55), they are 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 slowly 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, 35, 39, 54). Alignment of PV L1 sequences inclusive of BPCV1 and the subsequent phylogenetic analysis demonstrate that BPCV1 is most closely related to the members of the genus *Betapapillomavirus* (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 a descendant of the ancient common ancestor of both virus families. This represents the first report of a possible recombination between members of the families *Papillomaviridae* and the *Polyomaviridae*.

Alternatively, BPCV1 could be a descendant of a common ancestor of both the modern-day *Polyomaviridae* and *Papillomaviridae*. Despite not sharing major nucleotide or amino acid similarity, PVs and PyVs encode proteins sharing similar functions and domains. The actions of PV E7 and the 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 (11, 21). Therefore, although the 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 the two virus families (54). In the large T antigen tree (Fig. 2), BPCV1 appears as a close-to-root sequence, branching off from the root between the mammalian and avian PyVs. In contrast to the L1 analysis, this would be in agreement with the virus being a descendant of a common PV-PyV ancestor or at least having arisen as long ago as the two major phylogenetic clades of PyVs, in the earliest evolutionary history of the *Polyomaviridae*.

Due to the novel nature of this virus, we propose that it is inappropriate for BPCV1 to be classified as either a PV or a PyV, and it may in fact represent the first member of a new and yet-to-be-classified family of viruses. Not only is the detection of BPCV1 important for conservation efforts to prevent the extinction of the WBB, it will surely open discussion concerning theories of the evolution and divergence of virus families previously belonging to *Papovaviridae*, as well as the final classification of this virus.

ACKNOWLEDGMENTS

This project is funded by the Australian Research Council in partnership with Murdoch University and the Western Australian Department of Environment and Conservation (DEC) under Linkage Project LP0455050. Lucy Woolford is funded by the Lorna Edith Murdoch Veterinary Trust Scholarship and was assisted by the Murdoch Veterinary Trust Weston Fernie Scholarship. Laboratory bench work was performed both at the Western Australian State Agricultural and Biotechnology Centre (SABC), Perth, Australia, and at the Catholic University, Leuven, Belgium, where it was supported by Flemish Fund for Scientific Research (Fonds voor Wetenschappelijk Onderzoek [FWO]) grant G.0513.06 and by a postdoctoral fellowship of the Research Fund K.U. Leuven to Annabel Rector.

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:**3195–3200.
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:**5329–5334.
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:**540–552.
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, PA.
10. **Crandall, K. A., M. Perez-Losada, R. G. Christensen, D. A. McClellan, and R. P. Viscidi.** 2005. Phylogenomics and molecular evolution of polyomaviruses. *In* N. Ahsan (ed.), Polyomaviruses and human diseases, vol. 577. Springer, New York, NY.
11. **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.
12. **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.
13. **de Villiers, E., C. Fauquet, T. R. Broker, H. Bernard, and H. zur Hausen.** 2004. Classification of papillomaviruses. Virology **324:**17–27.
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 **2:**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, United Kingdom.
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, PA.
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. Characterization of two novel polyomaviruses of birds by using multiply primed rolling-circle amplification of their genomes. J. Virol. **80:**3523–3531.
26. **Kocher, T. D., W. K. Thomas, S. V. Meyer, S. V. Edwards, S. Paabo, F. X. Villablanca, and A. C. Wilson.** 1989. Dynamics of mtDNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA **86:**6196–6200.
27. **Kumar, S., K. Tamura, and M. Nei.** 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. **5:**150–163.
28. **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, PA.
29. **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.
30. **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, PA.
31. **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.
32. **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.
33. **Narechania, A., Z. Chen, R. DeSalle, and R. D. Burk.** 2005. Phylogenetic incongruence among oncogenic genital alpha human papillomaviruses. J. Virol. **79:**15503–15510.
34. **Nicholas, K. B., H. B. Nicholas, and D. W. Deerfield.** 1997. GeneDoc: analysis and visualization of genetic variation. EMBnet News **4:**1–4.
35. **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.
36. **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.
37. **Pipas, J. M.** 1992. Common and unique features of T antigens encoded by the polyomavirus group. J. Virol. **66:**3979–3985.
38. **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.
39. **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.
40. **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.
41. **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, p. 1–90.
42. **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, NY.
43. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
44. **Scherneck, S., R. Ulrich, and F. Feunteun.** 2001. The hamster polyomavirus—a brief review of recent knowledge. Viral Genes **21:**93–101.
45. **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, Australia.
46. **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.
47. **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.
48. **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.
49. **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, p. 47–68. *In* G. Gross and G. von Krogh (ed.), Human papillomavirus infections in dermatovenereology. CRC Press, Boca Raton, FL.
50. **Tachezy, R., G. Duson, A. Rector, A. B. Jenson, J. P. Sundberg, and M. Van Ranst.** 2002. Cloning and genomic characterisation of *Felis domesticus* papillomavirus type 1. Virology **301:**313–321.
51. **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.
52. **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.
53. **Tjian, R.** 1978. The binding site on SV40 DNA for a T antigen related protein. Cell **13:**165–179.
54. **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, United Kingdom.
55. **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:**2527–2531.
56. **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.
57. **Woolford, L., A. J. O'Hara, M. D. Bennett, M. Slaven, R. Swan, J. A. Friend, A. Ducki, C. Sims, S. Hill, P. K. Nicholls, and K. S. Warren.** Cutaneous papillomatosis and carcinomatosis in the western barred bandicoot (*Perameles bougainville*). Vet. Pathol., in press.
58. **Xia, X., and X. Xie.** 2001. DAMBE: software package for data analysis in molecular biology. J. Hered. **92:**371–373.
59. **zur Hausen, H.** 1996. Papillomavirus infections—a major cause of human cancers. Biochim. Biophys. Acta **1288:**55–78.