

Detection of novel polyomaviruses in fruit bats in Indonesia

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Abstract Bats are an important natural reservoir for a variety of viral pathogens, including polyomaviruses (PyVs). The aims of this study were: (i) to determine which PyVs are present in bats in Indonesia and (ii) to analyze the evolutionary relationships between bat PyVs and other known PyVs. Using broad-spectrum polymerase chain reaction (PCR)-based assays, we screened PyV DNA isolated from spleen samples from 82 wild fruit bats captured in Indonesia. Fragments of the PyV genome were detected in 10 of the 82 spleen samples screened, and eight full-length viral genome sequences were obtained using an inverse PCR method. A phylogenetic analysis of eight whole viral genome sequences showed that BatPyVs form two distinct

genetic clusters within the proposed genus *Orthopolyomavirus* that are genetically different from previously described BatPyVs. Interestingly, one group of BatPyVs is genetically related to the primate PyVs, including human PyV9 and trichodysplasia spinulosa-associated PyV. This study has identified the presence of novel PyVs in fruit bats in Indonesia and provides genetic information about these BatPyVs.

Introduction

Polyomaviruses (PyVs) are small DNA viruses with a genome of approximately 5 kbp. They belong to the family *Polyomaviridae*. The viral genome is composed of three parts: the noncoding control region (NCCR), the early

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region, and the late region. The NCCR controls bidirectional transcription from both the early and late promoters and also contains the origin of viral DNA replication. The early region contains genes coding for regulatory proteins, known as tumor antigens, including a large T-antigen (T-Ag) and a small t-antigen (t-Ag). The late region encodes the structural proteins VP1, VP2, and VP3. Some primate PyVs also encode a small highly basic protein known as agnoprotein.

With the development of molecular diagnostic techniques, new PyVs have been identified recently in various animals [7, 16, 22, 24]. The *Polyomaviridae* Study Group of the International Committee on Taxonomy of Viruses (ICTV) has recommended that family *Polyomaviridae* be divided into three genera [7]. The proposed genus of avian-hosted PyVs is named *Avipolyomavirus*, and the divergence of members of this genus from the mammalian-hosted PyVs is based on both biological and genomic differences [3, 7]. The two proposed genera of mammalian-hosted PyVs are designated *Wukipolyomavirus* and *Orthopolyomavirus*, and these genera are divided based on nucleotide sequence divergence [3, 7]. The genus *Wukipolyomavirus* includes four human viruses: WU polyomavirus (WUPyV), KI polyomavirus (KIPyV), human polyomavirus 6 (HPyV6), and human polyomavirus 7 (HPyV7), whereas the proposed genus *Orthopolyomavirus* includes a variety of PyVs from primates, humans, cattle, rodents, and bats. Some of these PyVs cause subclinical infections with life-long persistence in their natural immunocompetent hosts, but they can also reactivate and cause diseases in immunocompromised hosts [9, 10, 24].

Various PyV genomes have been detected in bats [3, 15, 22], which are known to harbor and transmit a variety of emerging viruses to other mammals [4, 12, 14]. The bat PyV (BatPyV) and primate PyV groups tend to form monophyletic groups in phylogenetic trees [22]. One group of BatPyVs is genetically related to the human Merkel cell PyV, which has been implicated in the pathogenesis of Merkel cell carcinoma [22]. These findings led us to hypothesize that BatPyVs may be important for the maintenance and transmission of primate PyVs, including human PyVs. In this study, to verify our hypothesis, we investigated the presence of PyVs in bats in Indonesia using molecular sequencing and phylogenetic analysis.

Materials and methods

Sample collection and DNA extraction

A total of 88 fruit bat specimens were collected from the Republic of Indonesia during 2012 and 2013. After euthanasia, a complete necropsy was performed on the

captured bats. Specimens including the spleen were obtained and stored on ice in the field and were later transferred to -80°C storage before further processing. Our research was performed in accordance with the ethical guidelines of the Animal Care and Use Committee of the Animal Teaching Hospital, Bogor Agricultural University. The protocols used were approved by the Animal Care and Use Committee of Veterinary Teaching Hospital, Bogor Agricultural University (permit number 05-2010 RSHP-IPB). All samples were exported with the permission of the Directorate General of Livestock and Animal Health Services, Ministry of Agriculture, Republic of Indonesia. DNA was isolated from each spleen using a QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA) or DNAzol Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

PyV PCR and identification of bat species

The total DNA isolated from bat spleen tissues ($n = 88$) was screened for the presence of PyV DNA using a nested, broad-spectrum, polymerase chain reaction (PCR) method with consensus degenerate primers targeted against a conserved region within the VP1 gene, using high-fidelity Taq DNA polymerase (Roche Diagnostics, Indianapolis, IN, USA) [6, 16, 24]. PCR products were visualized after electrophoresis using ethidium-bromide-stained 2 % agarose gels, and positive bands of the expected size were purified using a QIAquick Gel Extraction Kit (QIAGEN). The purified PCR products were sequenced in both directions, using the PCR primers and a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies). After sequencing, a homology search was performed using BLAST. The species of fruit bats were determined based on their morphological characteristics and sequence analysis of their mitochondrial 16S rRNA and mitochondrial cytochrome *b* genes [1, 19].

Complete genome of PyV

Ten of the samples of PyV obtained from the fruit bats were selected for full-genome sequencing. We applied two strategies: direct PCR amplification and rolling-circle amplification (RCA). The RCA was performed on samples that had a low yield upon PCR amplification using an Illustra TempliPhi DNA Amplification Kit (GE Healthcare UK Ltd, Buckinghamshire, England) [23]. The PCR primer sets were designed from the nucleotide sequence of VP1 as described above. The PCRs were performed using PrimeSTAR GXL DNA Polymerase (Takara Biochemical, Berkeley, CA, USA). The PCR products were cloned into the pUC118 vector using a Mighty Cloning Kit (Takara Biochemical), and sequences were determined using an Ion

PGM Sequencing 400 Kit (Life Technologies). The resulting reads were assembled *de novo* using CLC genomics 6.0. We obtained at least 400,000 reads (mean read length, 284 bp) for each BatPyV. Gaps and low-coverage regions were sequenced manually using the Sanger method. Prediction of splicing sites was performed using the NetGene2 server (<http://www.cbs.dtu.dk/services/NetGene2/>) [2, 5] and NNSplice server (http://www.fruitfly.org/seq_tools/splice.html) [17].

Phylogenetic analysis

The amino acid sequences associated with the following reference virus genes were obtained from GenBank: BatPyV2a (AT7), BatPyV2b (R266), BatPyV2c (A504), BatPyV3a (A1055), BatPyV3a (B0454), BatPyV4a (R104), BatPyV4b (C1109), BKPyV, LPyV, BPyV, ChPyV, Gg-gPyV1, GTM203 PteronotusPyV, HaPyV, HPyV9, HPyV12, JCPyV, KY156 Otomops PyV2, KY157 Otomops PyV1, KY270 Eidolon PyV1, KY336 Cardioderma PyV, KY369 Miniopterus PyV, and KY397 Chaerephon PyV1, MasPyV, MCPyV, MPtV, MPyV, MyoPyV, OraPyV1, OraPyV2, PtvPyV1a, PtvPyV2a, SV12, SV40, SqPyV, TSPyV and BPyV-2 (see Supplementary Table S1 for accession numbers). The selected viruses are the reported BatPyVs and are considered reference strains of the genus *Orthopolyomavirus* by the ICTV [11]. Multiple sequence alignments of the amino acid sequences of LT-Ag and VP1 were carried out using MEGA5 [21]. Bayesian phylogenetic analysis was performed using MrBayes software version 3.2.2 [18] with the WAG amino acid substitution model. The resulting trees were visualized with FigTree software, version 1.4.

Results

Detection of polyomavirus DNA in the spleens of fruit bats

We collected spleens from 88 fruit bats belonging to four different species (*Acerodon celebensis*, *Pteropus vampyrus*, *Pteropus* sp., and *Dobsonia moluccensis*) from three different districts (Paguyaman District, Surabaya District, and Yogyakarta District) in Indonesia. DNA was extracted from the spleens of these fruit bats and screened using a nested broad-spectrum PCR technique to detect the PyV VP1 region [6, 16, 24]. PCR products, approximately 250 bp in length, were detected in ten fruit bat samples (11.4 %), including one from a *Pteropus vampyrus* specimen captured in Surabaya District and five from *Dobsonia moluccensis*, two from *Acerodon celebensis*, and

two from *Pteropus* sp. specimens captured in Paguyaman District. The PCR products were analyzed using BLAST searches, which showed that all PCR products had at least a 76 % nucleotide sequence identity to the VP1 region of PyV sequences previously deposited in the GenBank database.

Viral genome analysis of BatPyVs

We attempted to identify the viral genomes of the 10 samples that were positive for the PyV VP1 region, using inverse primer sets that were designed based on the nucleotide sequence of the VP1 fragments. We amplified most of the viral genome, 5,000 bp, from each of five of the DNA samples. The other five DNA samples were used as a template for RCA [23], from which we successfully amplified the whole viral genome from three of these samples using an inverse PCR method. We failed to amplify the full PyV genome from the remaining two samples. In total, we managed to determine the complete viral genome sequences from eight of the samples. According to the ICTV, to establish a new PyV species, less than 81 % nucleotide sequence identity to other known PyVs is required, as determined through isolation of the whole genome and nucleotide sequencing [7]. Pairwise nucleotide sequence identities were calculated for comparisons with known reference PyV genome sequences (Table 1). Each of the eight genomes showed <81 % nucleotide sequence identity to the known reference PyVs, indicating that they represent novel PyV species. Because BatPyV1, BatPyV2, BatPyV3, and BatPyV4 have been reported previously [3, 15], these eight novel viruses were named BatPyV5a (detected in *Dobsonia moluccensis* specimens captured in Paguyaman District), BatPyV5b-1 (detected in *Pteropus vampyrus* specimens captured in Surabaya District), BatPyV5b-2 (detected in *Acerodon celebensis* specimens captured in Paguyaman District), BatPyV6a (detected in *Acerodon celebensis* specimens captured in Paguyaman District), BatPyV6b (detected in *Dobsonia moluccensis* specimens captured in Paguyaman District), BatPyV6c (detected in *Dobsonia moluccensis* specimens captured in Paguyaman District), BatPyV6d-1 (detected in *Pteropus* sp. specimens captured in Paguyaman District), and BatPyV6d-2 (detected in *Pteropus* sp. specimens captured in Paguyaman District). BatPyV6d-1 and BatPyV6d-2 had a 99.6 % nucleotide identity to each other, and BatPyV5b-1 shared 88.8 % nucleotide identity with BatPyV5b-2, suggesting that these sub-types belonged to the same strain. All viral genomes had the typical PyV genome organization, comprising one strand coding for regulatory proteins (t-Ag and T-Ag) and the other for structural proteins (VP1 and VP2/3) (Table 2).

Table 1 Pairwise nucleotide sequence identities of polyomavirus genomes

Virus	Percentage of nucleotide sequence identity							
	BatPyV5a	BatPyV5b-1	BatPyV5b-2	BatPyV6a	BatPyV6b	BatPyV6c	BatPyV6d-1	BatPyV6d-2
BatPyV5a		74.8	74.2	58	58	57.3	57.6	57.5
BatPyV5b-1	74.8		88.8	57.7	57.9	57.7	58.1	58
BatPyV5b-2	74.2	88.8		56.8	57.7	57.9	57.8	57.7
BatPyV6a	58	57.7	56.8		66.5	62.8	64	64
BatPyV6b	58	57.9	57.7	66.5		62.2	62.4	62.4
BatPyV6c	57.3	57.7	57.9	62.8	62.2		65.2	65.4
BatPyV6d-1	57.6	58.1	57.8	64	62.4	65.2		99.6
BatPyV6d-2	57.5	58	57.7	64	62.4	65.4	99.6	
JCPyV	55.3	55.7	55.3	55.7	54.9	57.8	55.6	57.4
BKPyV	56.3	55.3	55.3	57.7	55.7	55.2	56	56.6
MCPyV	59.8	58.7	58.9	55.6	54.6	56.7	54.4	54.2
TSPyV	62.7	61.3	61.6	57	56.6	57	57.1	57.1
SqPyV	57.4	57.7	57.7	64.7	63.1	62.7	63.2	63.2
BatPyV4a(R104)	59.8	59.1	59.1	55	55.5	55.9	55.4	55.3
KY336 <i>Cardioderma</i> PyV	57.9	56.8	58.3	53.5	54.8	54.3	53.9	53.9
BatPyV3a(A1055)	59.8	59	59.5	56.2	56.2	55.9	54.9	54.9
KY156 <i>Otomops</i> PyV2	60.6	58.6	58.7	56.3	56.8	56.3	55.5	55.5
BatPyV2a(AT7)	57.7	56.6	56.7	58.7	58.3	58.9	58.8	58.6
GTM203 <i>Pteronotus</i> PyV	57.2	56.2	56.6	58.5	58.4	59.5	57.8	57.6
MyoPyV	56.4	56.5	56.1	61.1	58.4	60.9	61.3	61.2
KY369 <i>Miniopterus</i> PyV	59.5	58	58.1	60.8	59.7	60.1	59.3	59.4

Table 2 Profile of PyV genomes identified from the spleens of fruit bats in Indonesia

Virus	Origin	Cluster	Genome size (nt)	ORF (aa)			
				t-Ag	T-Ag	VP1	VP2/3
BatPyV5a	<i>Dobsonia moluccensis</i>	D	5075	136	647	382	309
BatPyV5b-1	<i>Pteropus vampyrus</i>	D	5047	188	626	312	311
BatPyV5b-2	<i>Acerodon celebensis</i>	D	5040	199	634	378	200
BatPyV6a	<i>Acerodon celebensis</i>	E	5019	162	641	366	340
BatPyV6b	<i>Dobsonia moluccensis</i>	E	5039	162	652	368	334
BatPyV6c	<i>Dobsonia moluccensis</i>	E	5046	165	674	360	333
BatPyV6d-1	<i>Pteropus</i> sp.	E	4956	166	661	367	324
BatPyV6d-2	<i>Pteropus</i> sp.	E	4956	166	661	367	324

Genome organization of BatPyVs

ORFs present within the genome of PyVs are separated by NCCR into regulatory protein coding regions and structural protein coding regions [22]. Repeats of the DNA element GAGGC and its reverse complement GCCTC within the NCCR are regarded as T-Ag-binding sites, as shown previously [8]. The novel BatPyVs harbor four to six copies of these T-Ag-binding elements, and BatPyV6b possessed a palindromic octamer (Fig. 1),

which is an overlapping bidirectional T-Ag-binding element (GAGGCCTC) [8]. BatPyV6a, BatPyV6c, BatPyV6d-1, and BatPyV6d-2 had no GCCTC complements, and none of the novel BatPyVs had two repeats of GAGGC followed by a palindromic repeat of the GCCGTC complement (Fig. 1). The number and arrangement of T-Ag-binding elements in the novel BatPyVs were different from those of TsPyV, OraPyV1, and previously known BatPyVs. Interestingly, a high degree of similarity in the NCCR of the BatPyVs from cluster D

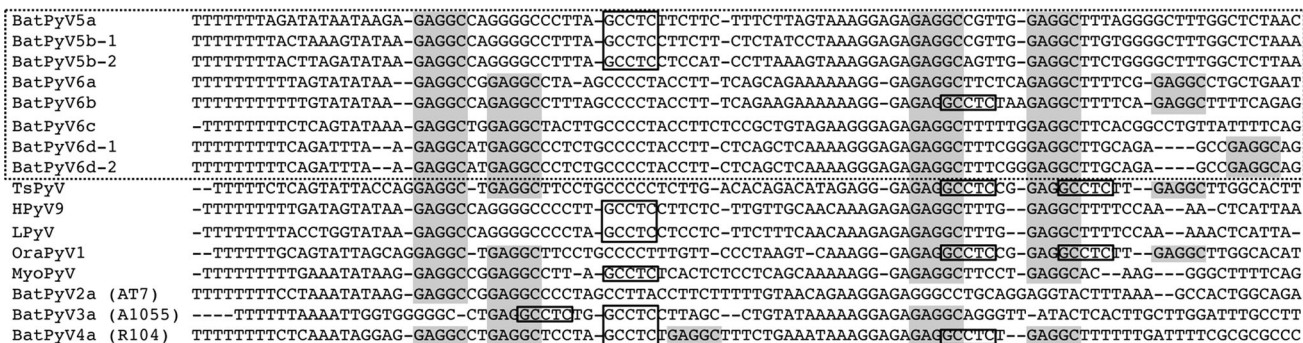


Fig. 1 Sequence alignment of the noncoding control region of selected PyVs. DNA sequences from the BatPyVs discovered in this study were compared with the NCCR of the other known PyVs using the Clustal W program, with additional manual adjustments. The

novel BatPyVs are boxed within a dotted line. T-Ag-binding elements (GAGGC or the complement GCCTC) are shaded in gray (GAGGC) and boxed (GCCTC)

(BatPyV5a, BatPyV5b-1, and BatPyV5b-2), HPyV9, and LPyV was shown, even though the NCCR is the most variable region of the PyV genome.

The T-Ag of PyVs exhibits a high degree of conservation within its functional domains, including the J domain and the retinoblastoma protein (Rb)-binding motif [8, 13]. As for TSPyV, HpyV9, LPyV, and OraPyV1, all of the T-Ags from the novel BatPyVs possessed a J domain with the highly conserved motif HPDKGG (Fig. 2). The T-Ag from BatPyVs of cluster D (BatPyV5a, BatPyV5b-1, and BatPyV5b-2) lacked an LXCXE Rb-binding motif, whereas the other novel BatPyVs contained this motif (Fig. 2).

Phylogenetic analysis

Phylogenetic analysis was performed on the basis of the aligned amino acid sequences of the LT-Ag and VP1 to investigate the evolutionary relationships of the novel BatPyV to other known PyVs (Fig. 3). To construct phylogenetic tree, we excluded members of the proposed genera *Avipolyomavirus* and *Wukipolyomavirus* because of their significant heterogeneity. It has been reported that there are three distinct well-supported genetic clusters of BatPyVs (A, B, and C) [3]. Based on the LT-Ag and VP1 phylogenies (Fig. 3), the novel BatPyVs formed two distinct genetic clusters: (i) BatPyV5a, BatPyV5b-1, and

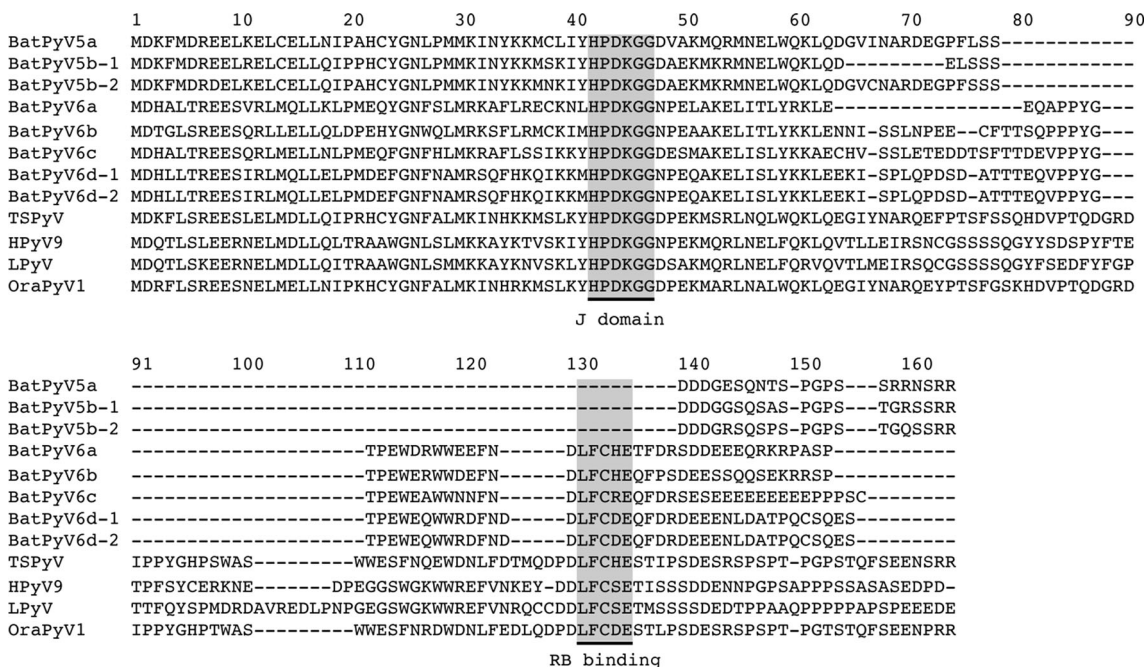


Fig. 2 Protein sequence alignment of the T-Ag N-terminal portions. The alignment was done using the Clustal W program, with additional manual adjustments. The regions shaded in gray regions indicate the J domain (HPDKGG) and the Rb-binding domain (LXCXE)

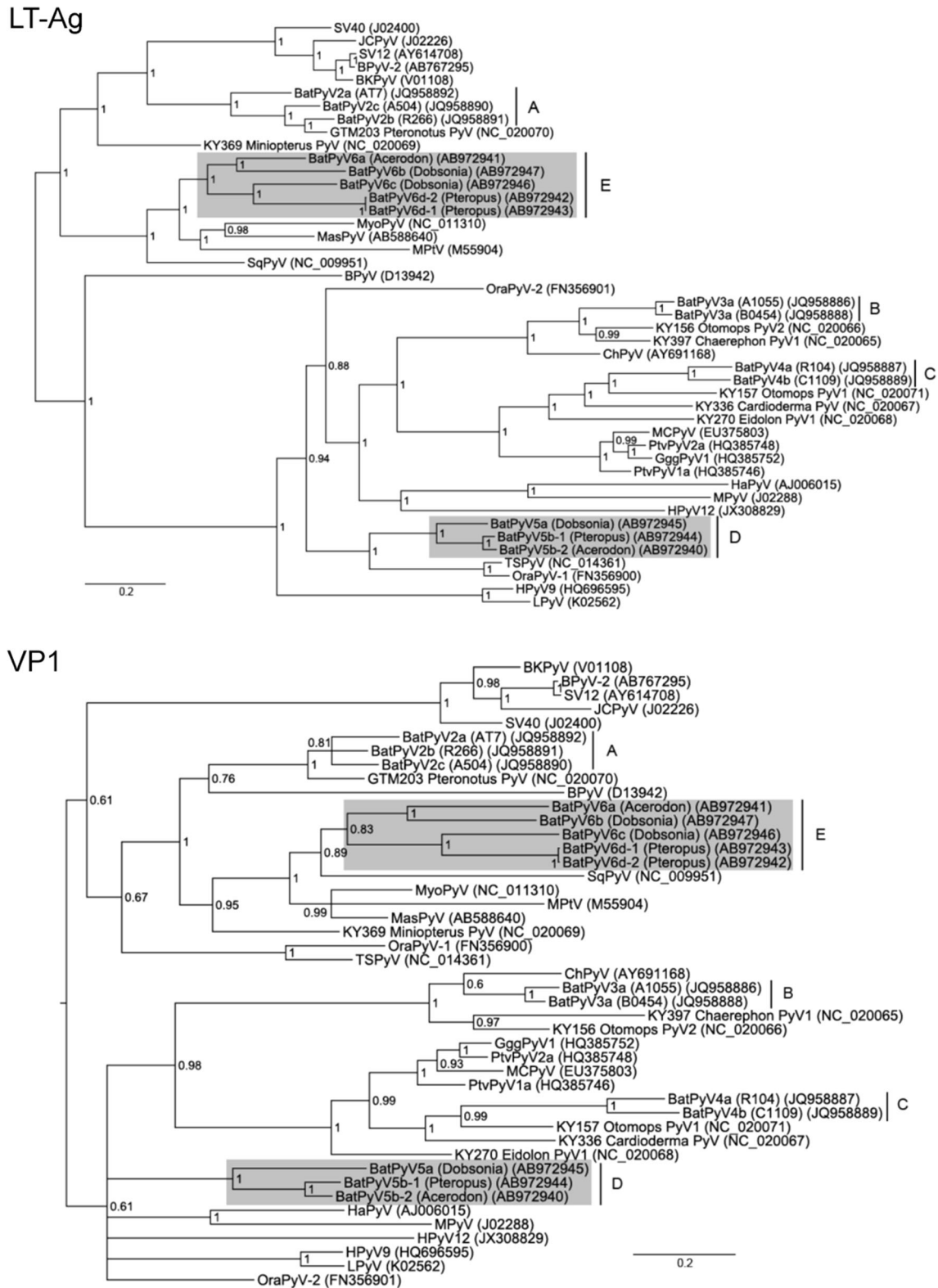


Fig. 3 Phylogenetic analysis based on the amino acid sequence of LT-Ag and VP1 of PyVs. Lineages leading to BatPyVs identified in this study are shaded in gray. Phylogenetic analysis was performed using MrBayes software version 3.2.2, with percentages indicated at

the nodes. GenBank accession numbers for the viruses used in the tree are shown in parentheses. Scale bars are in units of nucleotide substitutions per site

BatPyV5b-2 (cluster D); and (ii) BatPyV6a, BatPyV6b, BatPyV6c, BatPyV6d-1, and BatPyV6d-2 (cluster E). From the LT-Ag phylogeny, the BatPyVs from cluster E were related to PyVs from a variety of species, including monkeys (SqPyV), rodents (MPtV and MasPyV), and bats (MyoPyV). None of the BatPyVs from cluster D showed a relationship to known BatPyVs, whereas BatPyVs from cluster D were related to TSPyV and OraPyV1.

Discussion

In this study, we have identified eight novel PyV genomes in fruit bats from Indonesia. Phylogenetic analysis indicated that these viruses are divided into two clusters (cluster D and E). Cluster D BatPyVs are related to the primate PyVs, such as TSPyV and OraPyV1. Previous reports have shown that BatPyV clusters B and C and primate PyVs have relevance, as they tended to form monophyletic groups in phylogenetic trees [3, 22]. These relationships between BatPyVs and primate PyVs suggest that bats and primates may be closely tied in the PyV infectious cycle. TSPyV has been identified in human trichodysplasia spinulosa, which is a rare skin disease exclusively found in severely immunocompromised hosts [9, 20, 23]. The genome of the novel BatPyVs described herein contains common ORFs of known PyVs, such as T-Ag, t-Ag, VP1, and VP2/3, but not agnoprotein. The T-Ag of PyVs is a multifunctional protein composed of several functional domains [16]. The T-Ag interacts with molecular chaperones and Rb family proteins that regulate the cell cycle to replicate viral genome [8, 13]. The cluster D BatPyVs lack an Rb-binding motif (LXCXE), but they have a molecular chaperone-binding motif (the J domain: HPDKGG). The introns of the LT-Ag mRNAs from the cluster D BatPyVs contain the canonical dinucleotides GT and AG for splice donor and splice acceptor sites, respectively (Supplementary Table S2). The Rb-binding motif was not found in the region upstream of the splice acceptor sites. However, because splicing sites were analyzed using a splice-site prediction program, they need to be confirmed by analyzing mRNA in PyV-infected cells.

We found eight novel BatPyV genomes in spleen tissue from fruit bats using broad-spectrum PCR, but there were no noticeable gross pathological findings in the tissues of these fruit bats. Although an infection with any of these eight novel BatPyVs is likely to be asymptomatic in a healthy individual, as for other PyVs, this clearly requires further study.

In conclusion, we detected eight novel PyV genomes in fruit bats in Indonesia. This is the first study to identify the viral genome of PyV in fruit bats in Indonesia. Our findings are consistent with previous reports that transmission of

PyV occurs between bats and primates [3, 22]. Further epidemiological investigations are needed to determine the extent of PyV genetic variation in various mammalian species.

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