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Phylogenetics, evolution, and medical importance of polyomaviruses

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ABSTRACT

The increasing frequency of tissue transplantation, recent progress in the development and application of immunomodulators, and the depressingly high number of AIDS patients worldwide have placed human polyomaviruses, a group of pathogens that can become reactivated under the status of immunosuppression, suddenly in the spotlight.

Since the first description of a polyomavirus a half-century ago in 1953, a multiplicity of human and animal polyomaviruses have been discovered. After reviewing the history of research into this group, with a special focus is made on the clinical importance of human polyomaviruses, we conclude by elucidating the phylogenetic relationships and thus evolutionary history of these viruses. Our phylogenetic analyses are based on all available putative polyomavirus species as well as including all subtypes, subgroups, and (sub)lineages of the human BK and JC polyomaviruses. Finally, we reveal that the hypothesis of a strict codivergence of polyomaviruses with their respective hosts does not represent a realistic assumption in light of phylogenetic findings presented here.

1. Introduction

Polyomavirus represents the sole genus within the family *Polyomaviridae*. At present, 5 human and 16 non-human (12 mammalian and 4 avian) polyomavirus species are known (see Table 1). Most mammalian polyomaviruses have not been directly linked to a severe acute disease after natural infection of an immunocompetent host. Instead, inconspicuous primary infection results in lifelong persistence. Under immunosuppression, however, reactivation of the viruses can occur leading to several disease patterns (e.g., progressive multifocal encephalopathy, hemorrhagic cystitis, among others). Furthermore, most mammalian polyomaviruses exhibit transforming properties in cell culture and are able to induce malignant tumours after inoculation into non-permissive rodents. These two features make them an ideal tool for cancer research. In general, it is believed that mammalian polyomaviruses have a narrow host range. Polyomaviruses of birds stand in sharp contrast to the mammalian strains: they possess a high degree of pathogenicity especially in young animals and none exhibit tumourigenic properties (Johne and Muller, 2007; zur Hausen, 2008a).

2. Molecular biology of polyomaviruses

Polyomaviridae are nonenveloped viruses possessing icosahedral capsids consisting of 72 capsomers in a skewed lattice arrangement ($T = 7$). Each of the pentameric capsomers is assembled by five molecules of the VP1 protein and one molecule of either the VP2 or VP3 proteins (Cole and Conzen, 2001; Liddington et al., 1991; Stehle et al., 1996). The capsid encloses a circular double-stranded DNA genome of approximately 5100 nucleotides that is coated by the host-cell histones H2A, H2B, H3, and H4 (core nucleosome). The viral DNA and 24–26 core nucleosomes together constitute the minichromosome (Muller et al., 1978), the packaging of which requires a relative large capsid of about 40–45 nm diameter. All polyomaviruses display a similar genome organization consisting of three functional regions (Hou et al., 2005): two regions encode the early and late proteins and are separated by a third nucleosome-free non-coding region (NCR) that contains the origin of replication (ORI) and regulatory regions for early and late transcription (see Fig. 1). The latter shows multiple arrangements depending on the tissue source from which the virus was isolated, particularly for the human polyomaviruses JC (JCV) and BK (BKV). For the sake of clarity, only the regulatory part of BKV is exemplified here. The linear arrangement of sequences derived from urine specimens has been designated as the 'archetypal' structure (Imperiale and Major, 2007), with BKV strain WW being a typical representative (Knowles, 2001).

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Table 1
Polyomaviruses of mammals and birds (adapted from Johne and Muller, 2007; zur Hausen, 2008a).

Host species	Virus (abbreviation)	Genome size (bp)	Disease after natural infection	Reference
Human	BK virus (BKV)[a]	5133	Hemorrhagic cystitis[b], nephropathy[c]	Gardner et al. (1971)
	JC virus (JCV)[a]	5130	Progressive multifocal leukencephalopathy[d]	Padgett et al. (1971)
	KI virus (KI)	5040	Not known[e]	Allander et al. (2007)
	WU virus (WU)	5229	Not known[e]	Gaynor et al. (2007)
	MC virus (MCPyV)	5387	Merkel carcinoma	Feng et al. (2008)
Chimpanzee	Chimpanzee polyomavirus (ChPyV)	Partial sequence (VP1)[f]	Not known	Johne et al. (2005)
Rhesus monkey[g]	Simian virus 40 (SV40)[a]	5243	Not known	Sweet and Hilleman (1960)
Vervet monkey Chacma baboon[g]	Simian virus 12/Simian agent 12 (SA12)[a]	5230	Not known	Malherbe and Harwin (1963)
Baboon[g]	Baboon polyomavirus 2 (PpyV)[a]	Not sequenced	Not known	Gardner et al. (1989)
African green monkey[g]	B-lymphotropic polyomavirus (LPyV)[a]	5270	Not known	zur Hausen and Gissmann (1979)
Squirrel monkey	Squirrel monkey polyomavirus (SquiPyV)	5075	Not known	Verschoor et al. (2008)
Mouse	Murine polyomavirus (MPyV)[a]	5297–5307	Not known	Gross (1953)
	Murine pneumotropic polyomavirus/ Kilham Virus (MPtyV)[a]	4754	Not known (severe pneumonia in newborn mice)	Kilham and Murphy (1953)
Rabbit[g]	Rabbit kidney vacuolating virus (RKV)[a]	Not sequenced	Not known	Ito et al. (1966)
Hamster	Hamster polyomavirus (HaPyV)[a]	5366	Skin tumours	Graffi et al. (1967)
Rat	Athymic rat polyomavirus (Rat-PyV)[h]	Not sequenced	Sialoadenitis in athymic nude rats	Ward et al. (1984)
Cattle[g]	Bovine polyomavirus (BPyV)[a]	4697	Not known	Shah et al. (1977)
Parrot and other bird species	Budgerigar fledgling disease polyomavirus or avian polyomavirus (BFPyV/APyV)[a]	4981	Budgerigar fledgling disease	Bernier et al. (1981), Bozeman et al. (1981)
Goose	Goose hemorrhagic polyomavirus (GHPyV)	5256	Hemorrhagic nephritis and enteritis	Guerin et al. (2000)
Finch	Finch polyomavirus (FPyV)	5278	(Polyomavirus disease)	Johne et al. (2006)
Crow	Crow polyomavirus (CPyV)	5079	Not known	Johne et al. (2006)

[a] Species as listed in the VIIIth report of the ICTV (Hou et al., 2005).
[b] Seen in bone marrow transplant recipients.
[c] Seen in renal transplant recipients.
[d] Primarily seen in AIDS-patients, AIDS-defining disease.
[e] First reports indicated an association with acute respiratory tract diseases.
[f] Sequence information is available for viral capsid protein 1 (VP1) only.
[g] Contaminants in cell culture.
[h] Tentative species as listed in the VIIIth report of the ICTV (Hou et al., 2005).

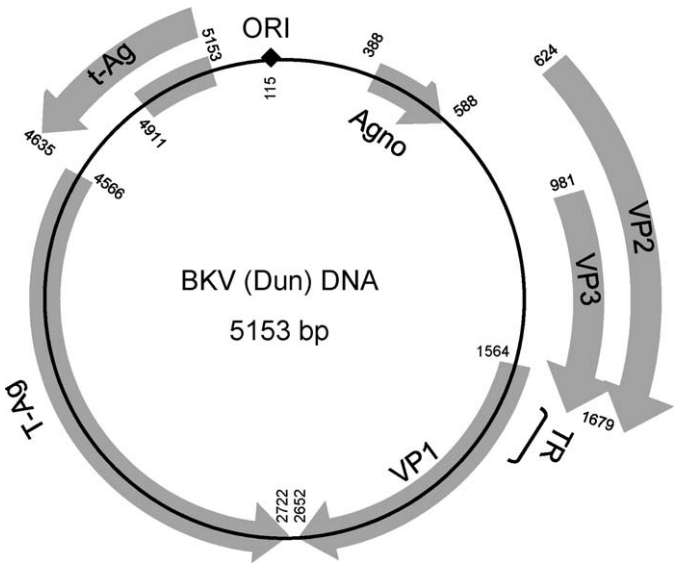

Fig. 1. Genome organization of polyomaviruses. All polyomaviruses display a similar genome organization, as exemplified by human polyomavirus BK (strain Dun). Six open reading frames (encoding the agnoprotein, the capsid proteins VP1, VP2 and VP3, early regulatory proteins large T-ag and small t-ag), the origin of replication (ORI) and the 287-nt typing region (TR) are indicated. Nucleotide numbers refer to Dun-numbering (Seif et al., 1979).

Furthermore, it has been suggested that the denotation 'archetype' should be used as a conceptual term to denote a prototypical structure (Krumbholz et al., 2008b; Takasaka et al., 2004; Yogo et al., 2008b). The archetypal configuration has been largely conserved during the evolution of BKV. The transcription control region (TCR) is arbitrarily divided into blocks designated P, Q, R, and S. Within these blocks, enhancer elements and transcription factor binding sites are located. Various rearranged TCRs can be generated during viral growth both *in vivo* and *in vitro*.

The early region consists of two overlapping open reading frames (ORFs) encoding the non-structural proteins large T- and small t-antigen (T-ag, t-ag). The early region of the mouse (MPyV) and hamster polyomaviruses (HaPyV) also encodes a middle T-antigen (see Table 2). The early transcripts are produced by alternative splicing from a common pre-mRNA before viral replication. Because both T-ag and t-ag use the same start codon, the N-terminal amino acids of these two proteins are identical. Another protein called 17kT is expressed from an alternatively spliced third early mRNA of simian virus 40 (SV40). While 131 amino acids of 17kT correspond to the N-terminus of T-ag, the four C-terminal amino acids are unique and encoded by a different reading frame (Zerrahn et al., 1993). 17kT seems to complement dnaj domain mutations of T-ag *in vitro*, and thereby restores the transforming abilities of T-ag (Boyapati et al., 2003). An alternative splice analog to SV40 17kT was found in the recently discovered Merkel cell polyomavirus (MCPyV) (Shuda et al., 2008). The "tiny t" of mouse polyomavirus (MPyV), T′135, T′136, and T′165 of JCV, and

Table 2
Polyomavirus proteins[a].

Protein/region	Function	References
Early coding		
Large T-antigen	Binding to viral promotor facilitates transcription of viral DNA Binding to cellular promotors leads to transactivation Binding to ORI DNA enables viral replication Interaction with key proteins of cell-cycle (e.g., p53, pRb) leads to abrogation of cell cycle arrest	Moens et al. (2007), White and Khalili (2006)
17kT of SV40	Potential role in transformation by the complementation of dnaj domain mutations in the large T-antigen	Boyapati et al. (2003), Zerrahn et al. (1993)
Middle T-antigen[b]	Facilitates host-cell transformation via binding and activation of tyrosine kinase pp60$^{c\text{-}src}$ and other members of the c-src family Serves as a substrate of pp60$^{c\text{-}src}$ Phosphorylated middle T-antigen activates phosphatidylinositol 3-kinases involved in intracellular signaling and mitogenesis	Benjamin (2001), Dilworth (2002)
Small t-antigen	Promotes cell-cycle progression and S-phase entry Facilitates host-cell transformation Binding and inhibition of protein phosphatase PP2A Activation of MAP kinase pathway Transactivation of cyclin promotors	Benjamin (2001), Khalili et al. (2008)
Late coding		
VPx—agnoprotein[c]	Modulation of viral replication and transcription Facilitates virion biogenesis Facilitates viral spreading	Moens et al. (2007), Okada et al. (2005), Resnick and Shenk (1986), Safak et al. (2001, 2002)
VP1	Major capsid protein Binds sialioglycoproteins (primary cellular receptors) and enables cell entry	Ahsan and Shah (2006), Benjamin (2001)
VP2	Minor capsid protein	Ahsan and Shah (2006)
VP3	Minor capsid protein Subset of VP2	Ahsan and Shah (2006)
VP4 of SV40[d]	C-terminal part of VP3 Involved in host-cell lysis	Daniels et al. (2007)
VP4 or homologous protein of birds[e]	Packaging of viral genome Induction of apoptosis	Johne and Muller (2007)

[a] Described functions apply to selected polyomaviruses; for details, please refer to the references given in the right column.
[b] MPyV and HaPyV only, reviewed by Dilworth (2002) and Scherneck et al. (2001).
[c] SV40, SquiPyV, BKV, and JCV only.
[d] Identified in SV40-infected cells, not incorporated in virions (Daniels et al., 2007).
[e] In polymoviruses of birds only, shares no apparent homology to VP4 of SV40 (Johne and Muller, 2007).

"mini T" of BKV are further examples for the expression of additional early mRNAs (Imperiale and Major, 2007; Riley et al., 1997). The late region of the genome codes for the structural proteins VP1, VP2, and VP3 (see Table 2). The latter shares part of its ORF with VP2, differing in the initiation codon. Four polyomaviruses – BKV, JCV, SV40, and squirrel monkey virus (SquiPyV) – also harbour sequence information for the so called agnoprotein (VPx), with the VPx ORF being located upstream of that for VP2/VP3. In human polyomavirus, VPx binds to heterochromatin protein 1α to induce its dissociation from the lamin B receptor, thereby promoting the nuclear export of progeny virus (Okada et al., 2005) through destabilization of the nuclear envelope. VPx of JCV acts to further modulate viral replication and transcription via its interaction with T-antigen and the cellular Y-box binding transcription factor (Safak et al., 2001, 2002). Finally, it has been demonstrated that VPx of SV40 facilitates viral spreading (Resnick and Shenk, 1986).

As with the early mRNAs, late transcripts are generated from a common pre-mRNA by alternative splicing. A recent study on SV40-infected cells revealed the existence of an additional viral protein called VP4 that appears to be involved in host-cell lysis but is absent in viral capsids (Daniels et al., 2007). Further polyomaviruses were found to possess a potential VP4 initiation codon at a similar position in their VP2 transcripts.

All known avian polyomaviruses have an additional ORF in the late region located close to the NCR. This location corresponds to that of the VPx ORF, but the resulting protein nevertheless shares no apparent sequence homology to VPx. More confusingly, this protein is designated as VP4 for avian polyomaviruses (APyV) such that care should be taken that it is not confounded with VP4 of SV40! A more detailed treatment on APyV VP4 and its splice variant VP4Δ can be found in the review of Johne and Muller (2007).

2.1. Lytic infection, persistence and oncogenic transformation by polyomaviruses

Polyomaviruses do not display absolute host-specificity, but do grow most efficiently in the cells of their respective host species (permissive cells). In general, these infections are non-oncogenic for their natural hosts (zur Hausen, 2008b). The viral replication is facilitated by host-cell enzymes (which are lacking in the viruses). The cellular S-phase environment exhibits the optimal condition for viral replication with its excess supply of these enzymes and deoxyribonucleotides. Because permissive cells normally persist in cell cycle arrest, the viral large T-antigen therefore acts to induce the expression of these enzymes by activation of E2F (Fig. 2). The strong apoptosis signal presented by the resulting unbalanced DNA

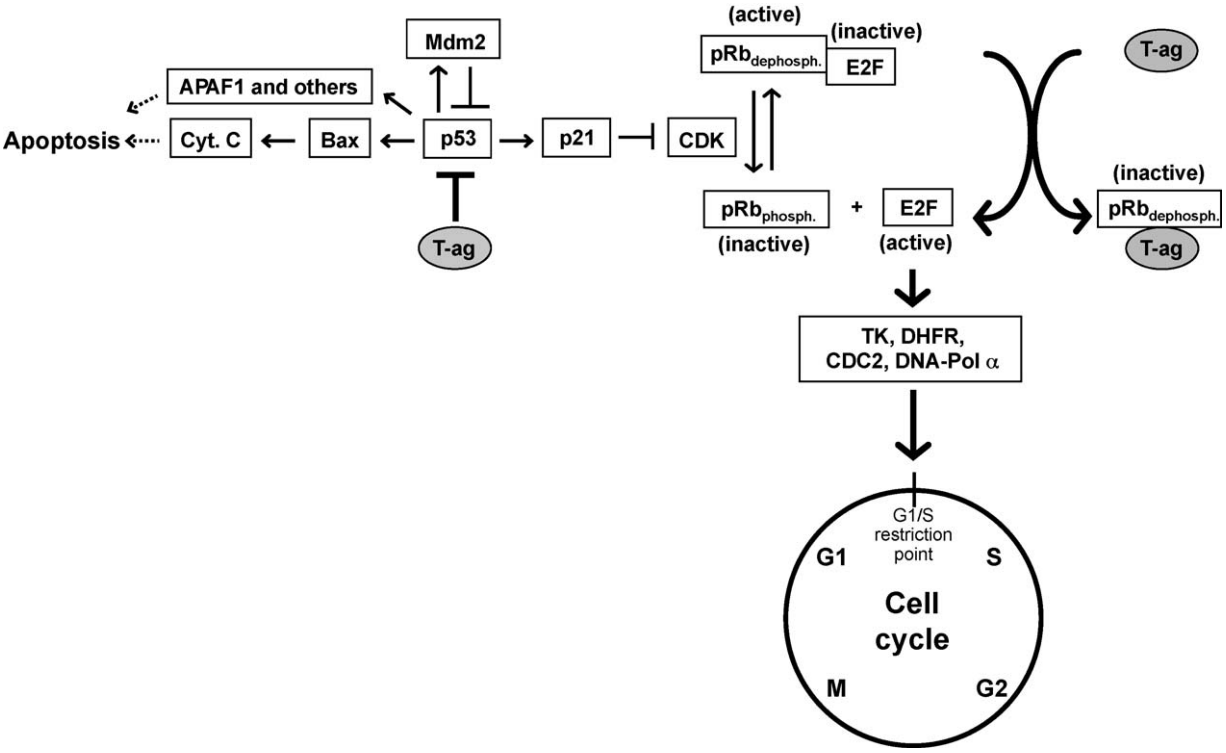

Fig. 2. SV40 T-ag-mediated cell transformation via inactivation of pRB and p53. In the non-infected cell, dephosphorylated (active) pRB controls proliferation by binding to and inactivating the transcription factor E2F, thereby keeping the cell in growth arrest. Under physiological conditions, E2F release is facilitated through the cyclin-dependent kinase (CDK)-mediated phosphorylation of pRB. The released E2F up-regulates the expression of enzymes necessary for DNA synthesis like thymidine kinase (TK), dihydrofolate reductase (DHFR), cell division cycle protein 2 (CDC2), and DNA polymerase α. The availability of these enzymes induces the S-phase of the cell. Cell cycle-independent replication of polyomaviruses requires the availability of dNTPs and enzymes during G1-phase. To overcome growth arrest, SV40 T-ag binds dephosphorylated pRB, thereby releasing E2F and creating a strong, unbalanced proliferation signal. Normally, such a signal would induce apoptosis by p53 through Bax-mediated cytochrome *C* release and through the transcriptional regulation of pro-apoptotic genes (illustrated here by APAF-1). However, SV40 T-ag binds p53 to prevent p53-induced apoptosis. In cells that are non-permissive for SV40, abortive virus replication leads to continuous proliferation without virion production and virus-induced lysis. Integration of the SV40 genome into the host-cell genome or the accumulation of errors due to the lack of p53-induced DNA repair together with suppressed p53-induced apoptosis may result in host-cell transformation.

synthesis is concomitantly suppressed by the binding of T-ag to the tumour suppressor protein p53. Theoretically, this process can result in host-cell transformation (i.e., the acquisition of expanded cell proliferation and an enhanced survival potential by a cell). However, polyomaviruses normally induce host-cell lysis (and therefore death) to release their progeny and to prevent their encapsulation in cellular membranes. The molecular basis of polyomavirus-induced host-cell lysis is poorly understood. Recently, the SV40 protein VP4 was described to be involved in this process when it was demonstrated that VP4 accumulates late in the viral replication cycle and is able to form hetero-oligomers with VP3, and possibly VP2. These complexes insert into the host-cell membranes to initiate cell lysis and viral release. Thereby, late expression of VP4 overcomes the T-ag-mediated prevention of apoptosis (Daniels et al., 2007).

During primary infection, not all cells perish by virus-induced lysis and primary infection is often followed by a subclinical lifelong persistence. The major sites of persistence for human polyomaviruses are the kidney, the central nervous system and the hematopoietic system. Little is known about the mechanisms promoting and perpetuating viral persistence and about the cellular factors and the instances that are responsible for the activation episodes of the asymptomatic persistent infection. Furthermore, it is still unclear whether or not the viruses enter a latent state or maintain a low level of viral gene expression and replication at these sites, although intermittent replication does occur as evidenced by periodic excretion of virus in the urine (Doerries, 2006; Jiang et al., 2009).

Oncogenic transformation is observed in cells that have underwent an abortive replication cycle (e.g., non-permissive cells). The molecular mechanisms underlying this process are complex and require the continuous expression of T-ag that interferes with key cellular targets. Among the latter, p53 and retinoblastoma protein pRb play a central role (Godefroy et al., 2006) (see Fig. 2). In general, polyomavirus-induced host-cell transformation requires the abruption of a complete viral replication cycle (e.g., after integration of the viral DNA into the cellular genome). Similarly, it also occurs in cells infected by a defective virus that cannot complete its lytic replication cycle.

The simian polyomavirus SV40 is undoubtedly the best studied polyomavirus that displays oncogenic transformation in non-permissive cells (Eddy et al., 1962) and the induction of malignant tumours in newborn hamsters (Girardi et al., 1962). Expression of large T-ag alone is sufficient to transform a variety of primary rodent cells (Bikel et al., 1987; Zhu et al., 1991, 1992) via binding and perturbation of the cellular tumour suppressor proteins p53 and pRb. Binding of pRb in particular prevents the inhibition of the E2F transcription factor, thereby up-regulating the transcription of cellular proteins necessary for DNA replication, nucleotide metabolism, DNA repair and cell cycle progression (the pleiotropic functions of E2F are listed in several recent reviews: Ahuja et al., 2005; Attwooll et al., 2004; Blais and Dynlacht, 2004; Bracken et al., 2004; Dimova and Dyson, 2005; Fan and Bertino, 1997; White and Khalili, 2006). Furthermore, S-phase entry is facilitated by T-ag-mediated transactivation of the cyclin A promoter. Potential escape from these processes on the part of the host cells by entering the apoptotic pathway via p53 is prevented by the T-ag-mediated inactivation of the latter (Ahuja et al., 2005). As a consequence, abolition of p53-controlled cell cycle arrest and

p53-induced apoptosis results in host-cell transformation. The interaction of T-ag with the insulin-like growth factor type I (IGF-I) signalling pathway represents a further mechanism promoting cellular transformation (Baserga et al., 1994). The transforming activity of T-ag can also be enhanced by mutations disrupting its replication and helicase activity (Manos and Gluzman, 1984; Prives et al., 1983; Small et al., 1982). The second early gene product small t-ag similarly modulates cell transformation through its negative regulation of the protein phosphatase 2A (PP2A) family of serine–threonine phosphatases that has been implicated in the regulation of numerous signalling pathways. This negative regulation leads to the stimulation of anti-apoptotic pathways and the induction of structural changes in the cytoskeleton (Sablina and Hahn, 2008).

The accidental exposure to SV40 in millions of poliovaccine recipients has resulted in intensive study of the question of whether or not SV40 can actually infect humans and thereby been responsible for several cancer entities. To date, however, neither epidemiological nor experimental data have provided convincing evidence for a role of SV40 in human malignancies. Furthermore, there is no clear evidence for the presence of specific SV40 antibodies in human sera (Poulin and DeCaprio, 2006; zur Hausen, 2008a).

Until recently, evidence of the tumourigenic potential of polyomaviruses was restricted to animal models only (Poulin and DeCaprio, 2006). However, the discovery of host cell-integrated polyomaviral DNA (Merkel cell polyomavirus, MCPyV) in extracts of Merkel cell carcinoma (Feng et al., 2008) is believed by many to represent the first human malignancy associated with a consistent presence of integrated DNA of a specific polyomavirus (Garneski et al., 2008; zur Hausen, 2008a). Furthermore, truncations were observed within the second exon of MCPyV T-ag that abrogate viral DNA replication capacity despite not altering its heat shock cognate 70 (Hsc70) and pRb binding (Garneski et al., 2008; Shuda et al., 2008). However, there are still arguments that question the involvement of MCPyV in Merkel cell cancer pathogenesis (Garneski et al., 2008).

3. Polyomaviruses in human and animals—a chronology of discovery

3.1. Human polyomaviruses

The first reports of the human polyomaviruses BKV and JCV were published coincidentally in 1971. BKV was cultivated from the urine of a kidney transplant recipient who developed ureteral stenosis (Gardner et al., 1971), whereas JCV was isolated from an immunocompromised patient who presented progressive multifocal leukencephalopathy (PML) (Padgett et al., 1971); the names of both viruses derive from the initials of the respective patient. Subsequent studies revealed that both viruses are ubiquitous and widely spread (seropositivity rates vary from 72 to 98%), with subclinical primary infection occurring in early childhood and normally leading to a lifelong persistence. Viral reactivation may occur as a result of immunosuppression but is also sometimes observed in immunocompetent hosts. In bone marrow recipients, BKV reactivation is linked to hemorrhagic cystitis, whereas severe tubulointerstitial nephritis and ureteric stenosis are causes of concern in renal transplant recipients (Hirsch, 2005). Several reports on disseminated BKV infection (e.g., meningitis, retinitis, pneumonia, or vasculopathy) involving organs in addition to the kidney or bladder suggest a wider cell tropism of this virus (Behre et al., 2008; Friedman and Flanders, 2006; Galan et al., 2005; Reploeg et al., 2001; Sandler et al., 1997). JCV is the etiologic agent of PML, a fatal demyelinating disease of the central nervous system caused by lytic infection of oligodendrocytes (see below). BKV and JCV are able to immortalize several cell-lines and inoculation of either into newborn animals can cause a variety of malignant tumours. Nevertheless, reports on the presence of JCV genomic sequences in human brain tumours continue to be highly controversial (zur Hausen, 2008a).

In recent years, large-scale molecular screening techniques have led to the identification of several new human polyomavirus species (Allander et al., 2007; Feng et al., 2008; Gaynor et al., 2007). Polyomaviruses KI (KIPyV) and WU (WUPyV) were detected in the nasopharyngeal aspirates obtained from patients presenting acute respiratory tract infections. Both are held to be more closely related to one other genetically than to either JCV or BKV (Allander et al., 2007; Gaynor et al., 2007). Although the initial reports of the viruses implicated their contribution to acute respiratory tract infections, recent studies have raised questions about this initial assumption (Abed et al., 2007; Norja et al., 2007). The most recently discovered human polyomavirus is MCPyV, which was reported using the digital transcriptome subtraction technique of RNA isolated from Merkel cell carcinomas (Feng et al., 2008). Merkel cell carcinoma is a rare neuroendocrinal tumour arising from mechanoreceptor Merkel cells and is one of the most aggressive forms of skin cancer (Becker et al., 2009b). MCPyV DNA was detectable in 8 of 10 Merkel tumours, mostly in a clonal integrated form (Feng et al., 2008). The presence of the MCPyV genome in the majority of Merkel cell carcinoma samples has been independently confirmed by other groups (Becker et al., 2009a; Garneski et al., 2009; Kassem et al., 2008).

3.2. Other mammalian polyomaviruses

Most polyomaviruses have been identified as contaminants in cell culture or cell-free preparations. In 1953, Ludwig Gross discovered the first polyomavirus while he was studying murine leukaemia virus. He observed that newborn mice inoculated with a contaminated preparation of this retrovirus developed not only leukaemia but also tumours of the parotid gland (Gross, 1953). Gross demonstrated that the agent responsible for these tumours was insensitive to heat treatment (65 °C) that otherwise inactivated murine leukaemia virus. Due to its ability to induce a variety of tumours (“polyoma”) in addition to parotid tumours in newborn mice, this virus was later named mouse polyomavirus (MPyV) (Stewart et al., 1958). Ever since, MPyV has served as a model agent of cell transformation and virus–host interaction in cancer research (Benjamin, 2001; Dulbecco and Vogt, 1960; Vogt and Dulbecco, 1960).

In parallel to BKV and JCV in humans, the murine pneumotropic polyomavirus (MPtyV) was coincidentally identified in the same year as MPyV (Kilham and Murphy, 1953). This virus can cause severe interstitial pneumonia in newborn mice, but normally leads to a persistent asymptomatic infection in immunocompetent adult animals.

SV40 was first identified as a contaminant of rhesus monkey kidney cell cultures used for poliovirus and adenovirus vaccine production between 1955 and 1963 (Sweet and Hilleman, 1960). In general, SV40 leads to a persistent asymptomatic infection of its natural host, the rhesus macaque (Minor et al., 2003), but has transforming properties in non-simian cell cultures (Eddy et al., 1962). Study of SV40 has led to fundamental insights into cell biology and genetics (see above) and has significantly improved our knowledge of (i) DNA and nucleosome structure, (ii) eukaryotic DNA replication and gene expression, and (iii) DNA transformation and oncogenesis (Imperiale and Major, 2007; Yaniv, 2009). In addition, the SV40 genome was the first viral genome to have been characterised physically, occurring soon after the discovery of restriction enzymes (Danna et al., 1973), and was the first eukaryotic virus to have its genome cloned and completely

sequenced (Fiers et al., 1978; Jackson et al., 1972; Reddy et al., 1978).

The next virus to be discovered, simian agent 12 (SA12), was isolated from kidney cell preparations of a vervet monkey (Malherbe and Harwin, 1963). A subsequent serological study, however, suggested that the Chacma baboon is the preferred natural host (Braun et al., 1980).

Despite its relative age, little information is available for the rabbit kidney vacuolating (RKV) virus, which was first isolated as a contaminant in some isolated batches of Shope's rabbit papillomata (Hartley and Rowe, 1964).

Hamster polyomavirus (HaPyV) was originally described in 1967 as an agent associated with skin epitheliomas of Syrian hamsters (Graffi et al., 1967). Soon thereafter, it was demonstrated that injection of epithelioma extracts into newborn hamsters can lead to the development of leukemias and lymphomas (Graffi et al., 1968a,b).

A further mammalian virus was isolated in 1974 from kidney cell cultures of the stump-tailed macaque (*Macaca arctoides*) and therefore initially called the stump-tailed macaque virus (STMV). However, subsequent serological studies indicated a bovine rather than a monkey origin of these isolates as a result of the contaminated bovine serum used in the cell cultures. Hence, the CK isolate of STMV was renamed subsequently as bovine polyomavirus (BPyV). A further study indicated that BPyV is a frequent contaminant of bovine serum, but has no apparent clinical significance for bovids (Schuurman et al., 1991).

The B-lymphotropic or African green monkey polyomavirus (LPyV) was isolated from a lymphoblastoid B-cell line obtained from an African green monkey (zur Hausen and Gissmann, 1979). The natural host of LPyV and its clinical relevance remain unknown.

Rat polyomavirus antigen (Rat-PyV) was found using immunohistochemistry in athymic nude rats presenting parotid sialoadenitis (Ward et al., 1984). No sequence information is available for this virus.

The presence of baboon polyomavirus 2/polyomavirus papionis (PPyV) was demonstrated in fluids from baboon kidney cell cultures. Serological data suggest that PPyV circulates independently from SA12 in baboons (*Papio anubis*) (Gardner et al., 1989). As for Rat-PyV, no sequence data are available for PPyV.

Using a broad-spectrum nested PCR approach, a chimpanzee polyomavirus (ChPyV) was detectable in the faeces of a juvenile chimpanzee that presented symptoms of severe diarrhoea. However, because this animal also was tested positive for rotaviruses and *Salmonella* sp., it remains unclear whether or not ChPyV represents a definite dysentery pathogen. To date, only sequence data for VP1 is available for this virus (Johne et al., 2005).

Finally, the first polyomavirus of a New World primate, SquiPyV, was described after its DNA was extracted from spleen tissue and frozen blood of a Bolivian squirrel monkey (*Saimiri boliviensis*). Its importance as a pathogen is unclear (Verschoor et al., 2008).

3.3. Polyomaviruses of birds

In 1981, budgerigar fledging disease, a generalised inclusion body disease characterised by hepatitis, ascites, hydropericardium and high mortality rates in young budgerigars (*Melopsittacus undulates*) was linked to a polyomavirus (Bernier et al., 1981; Bozeman et al., 1981). Because of the typical disease pattern in budgerigars, the International Committee on Taxonomy of Viruses recommended the designation of the virus as budgerigar fledging disease virus (BFPyV). However, in consideration of the broad host range – birds other than budgerigars also seem to be susceptible to the virus – the designation avian polyomavirus (APyV) is also, and more commonly, used (Johne and Muller, 2007).

Goose hemorrhagic polyomavirus (GHPyV) is the etiological agent of hemorrhagic nephritis and enteritis of geese (Guerin et al., 2000), where the clinical picture is characterised by hemorrhagic tubular necrosis and subcutaneous edemas. In Germany, a high seroprevalence rate was demonstrated even in asymptomatic stocks (Johne and Muller, 2007).

Finally, the application of a broad-spectrum PCR approach led to the description of two novel polyomaviruses of birds in 2006 (Johne et al., 2006): finch polyomavirus (FPyV) from a deceased bullfinch and crow polyomavirus (CPyV) from a dead crow. Only a few data on the clinical importance of both viruses are available (Johne and Muller, 2007; Wittig et al., 2007).

4. Medical importance of human polyomaviruses: diagnostic and therapeutic approaches to BKV and JCV reactivation in the immunocompromised host

4.1. Serological tools

Humans infected with BKV or JCV produce VP1-specific antibodies. Certain epitopes are associated with hemagglutination and cellular binding. Thus, hemagglutination inhibition assays are the method of choice to measure antibody titres to BKV and JCV (Hamilton et al., 2000), although neutralisation assays and immunoassays using virus-like particles are also used to measure antibody levels in human serum (Viscidi and Clayman, 2006).

However, the use of serology in the diagnosis of BKV/JCV reactivation-induced diseases is questionable in the routine diagnostic setting because, as mentioned above, primary infection typically occurs in early childhood and leads to a lifelong persistence, with seropositivity rates ranging from 72 to 98% (Dörries, 2004). By contrast, serological methods do play an important role in epidemiological surveys (Flaegstad et al., 1989; Knowles et al., 2003; Stolt et al., 2003). Here, four BKV serogroups are distinguishable: BKV prototype (I), BKV SB (II), BKV AS (III) and BKV IV (Knowles et al., 1989). All JCV strains are members of a single serotype (Major, 2001).

4.2. Histological and cytological findings in BKV reactivation

BKV associated nephropathy (BKVAN) is emerging as an important cause of renal allograft dysfunction. BKVAN is suspected in renal transplant patients showing a rise in serum creatinin levels during routine follow-up. The diagnosis is typically confirmed by histological examination of kidney biopsies (Egli et al., 2007). However, there is an increasing interest in the use of less invasive diagnostic methods such as urine cytology or quantification of viral load in blood and urine.

Urine shedding of so called "decoy cells" (epithelial cells with intranuclear viral inclusion bodies that are best identified in alcohol fixed Papinocolaou stained cytocentrifuged urine; Singh et al., 2006) is potentially indicative of BKV reactivation in the urothelium. However, whereas a lack of decoy cells in the urine has a negative predictive value of 99% for post-transplant associated BKV nephropathy, the positive predictive value is low. Thus, although urine cytology plays an important role in the screening of renal allograft recipients, it alone is not sufficient for the diagnosis of BKVAN (Vats et al., 2006).

4.3. Recent developments in diagnostics and therapy of BKV related diseases

The relation between the level of BKV replication, the development of BKVAN, and graft failure is poorly understood (Funk et al., 2007). The determination of BKV replication in the urine has become the most pivotal test to exclude BKVAN. In

patients with BKV viruria, plasma loads exceeding 10,000 copies/ml permit a presumptive diagnosis of BKVAN that needs to be confirmed subsequently by biopsy. In general, screening is recommended every 3 months during the first 2 years after transplantation, when allograft biopsies are performed for any reason, or when allograft dysfunction occurs (Egli et al., 2007). Carefully balanced reduction of immunosuppression plays an important role in BKVAN treatment. Furthermore, the antiviral drug cidofovir shows *in vitro* activity against murine polyomaviruses and has been used in several clinical trials (Trofe et al., 2006). Further studies have investigated BKVAN treatment with leflunomide, intravenous immunoglobulin, and fluoroquinolones (Trofe et al., 2006).

BKV-induced hemorrhagic cystitis (HC) in bone marrow transplant (BMT) recipients usually occurs in the post-engraftment period. BK viruria has been demonstrated to precede or coincide with disease onset and HC occurs four times more frequently in patients who excreted BKV than in those who did not. Recent data indicate that HC is associated with persistent high-level BKV viruria. Additional factors may also play a role in pathogenesis because 40–50% of transplant recipients exhibit a persistent BK viruria without developing HC. Current treatment of HC is supportive and accompanied by interventions that are designed to control bleeding. Specific prophylactic and therapeutic approaches are continuing objects of research. In the absence of intervention protocols of proven benefit, screening for BKV viruria in a clinical routine setting is not generally recommended (Dropulic and Jones, 2008; Egli et al., 2007).

4.4. Diagnostic and therapeutic approaches in PML

PML is a demyelinating disease of the CNS caused by a lytic infection of oligodendrocytes by JCV and characterised by symptoms including hemoplegia, monoplegia, akinesia, visual disturbances, diplopia, and dementia. It usually develops only in individuals with a severely compromised immune system. PML was rare before the emergence of HIV, but now affects ~5% of HIV-infected patients and is considered to be an AIDS-defining disease (Khalili et al., 2006). The incidence of PML complicating HIV/AIDS is higher than that of any other immunosuppressive disorder. This may be explained by any combination of (i) the degree and duration of cellular immunosuppression in HIV/AIDS, (ii) impaired JCV-specific CD4 T-cell responses, (iii) the erosion of the blood/brain barrier that facilitates the entry of B-lymphocytes infected with JCV, or (iv) molecular mechanisms whereby HIV-1 promotes JCV gene expression and participates in the pathogenesis of PML (Khalili and White, 2006). However, recent research indicates that host immune status alone cannot explain the incidence of PML fully (Sunyaev et al., 2009). Instead, the analysis of JCV sequences isolated from hosts with PML revealed a unique subset of common mutations in that portion of the VP1 gene encoding the binding site for sialic acid, the receptor used by JCV for cell infection. Sunyaev et al. (2009) held these mutations to have arisen via positive, adaptive evolution and to act to change the specificity of JCV for its cellular receptor(s).

Currently, there is no effective treatment for PML. However, the introduction of highly active antiretroviral therapy (HAART) for AIDS treatment has altered the clinical picture of HIV/PML, and the mortality rate has dropped from 90% to about 50% during the first 3 months of treatment (Khalili and White, 2006). Improved antiretroviral therapy has also influenced the diagnostic procedures. Initially, the diagnosis of PML was confirmed by brain biopsy in patients with appropriate neurological and neuroradiological features before being superseded by PCR-based detection of JCV DNA in the cerebrospinal fluid. In the era of HAART, however, the sensitivity of PCR has significantly decreased in treated patients such that brain biopsy may again be necessary to conclusively confirm the diagnosis (Koralnik, 2006).

5. Phylogenetics and evolution of polyomaviruses

5.1. Genetic subclassification of human polyomaviruses BKV and JCV

The major capsid protein VP1 is responsible for the antigenic variability among BKV isolates (Jin et al., 1993b). Although a high degree of similarity generally exists across the entire coding region of VP1 (>95% similarity among the ~1089 nucleotides/363 amino acids across all BKVs), the similarity in the region spanning amino acid residues 61–83 is only 61–70%. It is thought that these amino acids constitute the epitope responsible for serological subtyping. Indeed, the four recognised BKV serogroups I–IV (Jin et al., 1993b; Knowles et al., 1989) correlate with the division of BKVs into four major subtypes based on genotyping of this subgenic region (Jin et al., 1993b). However, genotyping based on partial VP1 sequences can result in low bootstrap support values (Krumbholz et al., 2006). Therefore, it has been suggested to instead use the entire VP1 sequence or even a concatenated data set consisting of several protein encoding sequences (Krumbholz et al., 2008a). By contrast, another recent study recommends the use of T-ag for unambiguous genotyping and the use of single nucleotide polymorphisms within the T-ag sequence in particular for rapid genotyping of BKV (Luo et al., 2009).

The regional distribution of the BKV subtypes has been established in several studies (Agostini et al., 1995; Baksh et al., 2001; Chen et al., 2006; Di Taranto et al., 1997; Ikegaya et al., 2006; Jin et al., 1993a, 1995; Krumbholz et al., 2006; Takasaka et al., 2004). Subtype I is the most prevalent, followed by subtype IV, with subtypes II and III occurring less frequently. With the exception of Japan, BKV subtype IV was previously found to be prevalent in East Asia (Chen et al., 2006); however, evidence exists that the prevalence of this subtype has been underestimated in other regions (Zheng et al., 2007). Subtype I has been subdivided further into the three subgroups Ia, Ib and Ic based on analyses of 121 sequences derived from urine specimens from Japanese renal and bone marrow transplant recipients (Takasaka et al., 2004). A regional distribution of these subgroups is also apparent: subgroup Ic is prevalent in Japan, Ib is widespread in European countries including Germany (Ikegaya et al., 2006; Krautkramer et al., 2009; Krumbholz et al., 2006; Takasaka et al., 2004), and Ia is prevalent in Africa (Zheng et al., 2007). Subtype Ib has recently been further subdivided into Ib-1 and Ib-2 (Zheng et al., 2007). Six subgroups of subtype IV have also been identified, each showing a close relationship to population geography. It was concluded that the subtype IV now circulating in human populations is derived from a virus that infected ancestral Asian populations (Nishimoto et al., 2007). Finally, it has been hypothesised that BKV has co-migrated with human populations in general (Zhong et al., 2009).

The diversity of the different BKV strains has implications for molecular diagnostics. For example, major mismatches within primer and probe sequences have been identified in up to 30.7% of known BKV strains (Luo et al., 2008), a general result which can lead to an underquantification of viral load or even to false-negative PCR results (Hoffman et al., 2008; Luo et al., 2008). Because the regions encoding VPx or VP2 exhibit the lowest degree of sequence variation for all viral genes across BKV, these ORFs might serve as preferred target sites for diagnostic PCR. However, their utility is limited currently by the lack of sequence data compared to those available for the VP1 ORF. A similar disproportion in the available sequence data also applies for the large T-antigen ORF, which is favoured as a PCR target site by some authors (Hirsch et al., 2001; Limaye et al., 2001). Thus, if only from a diagnostic perspective, there is an increasing interest in the

generation of complete sequence data (Hoffman et al., 2008; Luo et al., 2008).

All JCV strains belong to a single serotype (Major, 2001). Based on genetic variations, three JCV 'superclusters' (A, B, and C) are distinguishable (Sugimoto et al., 2002). Type A is distributed throughout Europe, type B throughout Asia and Africa with a minor subtype found in Europe, and type C in West Africa (Yogo et al., 2004). Types A and B have each been split further into a number of 'subordinated lineages' and 'sub-lineages' (16 lineages, 27 sub-lineages as of 15th of November 2008: Yogo et al., 2008a). A uniform nomenclature has yet to be adopted and several competing proposals exist at present (e.g., European subtype EU-a vs. Type 1, African subtype Af1 vs. Type 6: Jobes et al., 1998; Sugimoto et al., 1997).

Several JCV (sub)lineages (=genotypes) occupy distinct geographical domains, a finding that in combination with the worldwide distribution of JCV, its preferred transmission within families and its presumed genetic stability made JCV a candidate for tracing human migrations (Agostini et al., 1997; Pavesi, 2004, 2005; Sugimoto et al., 1997). Such an approach is based on the assumption of codivergence of JCV with humans, but a recent study has cast doubt on the appropriateness of this scenario. A reconciliation analysis of phylogenetic trees of human and JCV populations provided no evidence for a strict codivergence: although parts of the JCV phylogeny hint at codivergence, the phylogenies of several subtypes do not match the history of human populations (Shackelton et al., 2006). By contrast, another recent study did observe a correlation in geographical distribution patterns between human Y-chromosome haplogroups (i.e., clusters of closely linked DNA polymorphisms inherited as a unit) and JCV genotypes (Yogo et al., 2004), meaning that the debate regarding possible JCV-human codivergence remains open. It may be that codivergence is present only at local scales, as also found by Shackelton et al. (2006), but not globally across all JCV subtypes and human populations.

5.2. Molecular evolution of polyomaviruses—evidence for codivergence with hosts?

Among viruses, *Herpesvirales*, an order of large DNA viruses, are probably the best-studied example demonstrating codivergence with their hosts. In particular, significant evidence exists that the three distinct families of herpesvirus – *Herpesviridae*, containing viruses with mammals, birds and reptiles as their natural hosts; *Alloherpesviridae*, containing viruses of fish and amphibians and *Malacoherpesviridae*, consisting of a single invertebrate herpesvirus (Davison et al., 2009) – have a common origin and phylogenetic analyses of mammalian herpesviruses reveal a synchronous evolution of virus and host lineages over long timespans (McGeoch et al., 2006).

In polyomaviruses, the idea of potential host-dependent evolution was first introduced by Soeda et al. (1980) based on the congruence they found between the phylogenetic trees of the T-ag, t-ag and VP2/3 genes of MPyV, SV40, and BKV and those of their respective hosts (i.e., mouse, monkey, and man, respectively) as derived from globin sequences and fossil data. The codivergence hypothesis received further support with the inclusion of HaPyV and JCV and their respective hosts in the analyses (Shadan and Villarreal, 1993). However, the phylogenetic positions of APyV and the MPtyV, which are both highly lethal in young animals, were found to be incongruent with the host codivergence model in the same study. It was hypothesised at the time either that acute disease might disrupt the linkage between the virus and the host or that these non-kidney-infecting strains comprise a completely different viral lineage from the remaining forms (Shadan and Villarreal, 1993). However, our phylogenetic data do not support the latter hypothesis, with MPtyV clearly clustering with the other mammalian polyomaviruses (see Fig. 3 and supplementary Fig. 1). Furthermore, Shadan and Villarreal (1993) also suggested that maintaining a persistent subclinical infection is a normal and important biological strategy for the small DNA virus families such that lethal disease may be an exception. They further argued that a greater viral genetic diversity seems to be associated with acute disease. Finally, the aberrant expression of the host-cell control proteins p53 and pRb to enable high virulence was also discussed as an alternative explanation of the apparent non-codivergence events (Shadan and Villarreal, 1993).

The question of codivergence in polyomaviruses was recently revisited by Perez-Losada et al. (2006). Based on their investigations of 72 complete polyomavirus genomes, the codivergence hypothesis was largely upheld. In particular, they suggested that different viral life strategies (i.e., silent primary infection vs. symptomatic infection) can be accommodated with the hypothesis of polyomavirus host codivergence (Perez-Losada et al., 2006). Moreover, avian polyomaviruses were found to be clearly distinct from mammalian ones, with the inferred monophyly of each group supporting former, unofficial suggestions to designate each as distinct subgenera (Johne and Muller, 2003; Stoll et al., 1993).

By contrast, Perez-Losada et al. (2006) postulated host-switching events for several polyomaviruses to explain the observed inconsistencies between the host and viral trees. Notably, rodent and simian polyomaviruses were each found to be polyphyletic, but it was hypothesised that this finding might originate from differences in their tissue preferences and/or pathogenicity.

Additional support for codivergence comes from the apparently narrow host range of mammalian polyomaviruses, which implies a possible linkage to the molecular processes of host speciation. Molecular evidence for this hypothesis is provided from studies on MPyV and SV40 demonstrating that the host-cell source of the DNA polymerase α–primase complex plays an important role in discriminating between SV40 and MPyV T-ag-dependent replication of their cognate DNA *in vitro* (Murakami et al., 1986). Whereas cell extracts prepared from HeLa cells in the presence of SV40 T-ag replicated noncognate MPyV DNA poorly, the addition of purified DNA polymerase α–primase complex isolated from mouse cells enabled HeLa cell extracts to replicate MPyV DNA with the same efficiency as mouse cells (Murakami et al., 1986). Further experiments suggested that the interaction of mouse DNA primase with MPyV T-ag enables this species-specific initiation of viral DNA synthesis (Eki et al., 1991).

It must be pointed out, however, that the codivergence hypothesis in polyomaviruses has been based on comparatively few viral species to date. The continued discovery and phylogenetic analysis of new polyomavirus species is providing mounting evidence of non-codivergence events with the hosts (compare the studies above). For example, our results (Fig. 3 and supplementary Fig. 1) reveal that the newly described human polyomaviruses WU, KI and MCPyV clearly cluster apart from BKV and JCV (Allander et al., 2007; Feng et al., 2008; Gaynor et al., 2007). Likewise, the phylogenetic relationships of polyomaviruses recently isolated from Old World monkeys, chimpanzee, a New World monkey and rodents reveal similar inconsistencies (Johne et al., 2005; Verschoor et al., 2008). Our trees do show apparent avian and mammalian polyomavirus clades, but this is due to a subjective rooting of the trees in the absence of any outgroups. (Both groups, strictly speaking, are clans sensu Wilkinson et al., 2007.) With the exception of the T-ag gene, the two sets of viruses also do not show the high level of divergence that would be expected if codivergence has occurred given the comparatively deep divergence between birds and mammals.

Statistical testing of the codivergence hypothesis tends to bear these concerns out, at least at the level of the different

polyomavirus species. The crossing lines in the tanglegram in Fig. 4 linking a species-level polyomavirus tree with that of their hosts reveal a large incidence of non-codivergence events. For this tree, codivergence is rejected at the 0.05 level (global ParaFit = 114177153.0, $P = 0.49460$) based on the ParaFit test (Legendre et al., 2002). Moreover, none of the individual links in the tanglegram shows significant codivergence signal (see supplementary Table 2).

The rejection of global codivergence between the different polyomavirus species and their hosts does not automatically negate the possibility of more restricted codivergence events (see above for JCV and also Gottschling et al., 2007). For instance, a ParaFit analysis of all the sequences in Fig. 3d (and not just the individual species) does reveal significant codivergence (global ParaFit = 33753018760.0, $P = 0.00003$), albeit largely due to the large BKV and JCV samples, but also with significant

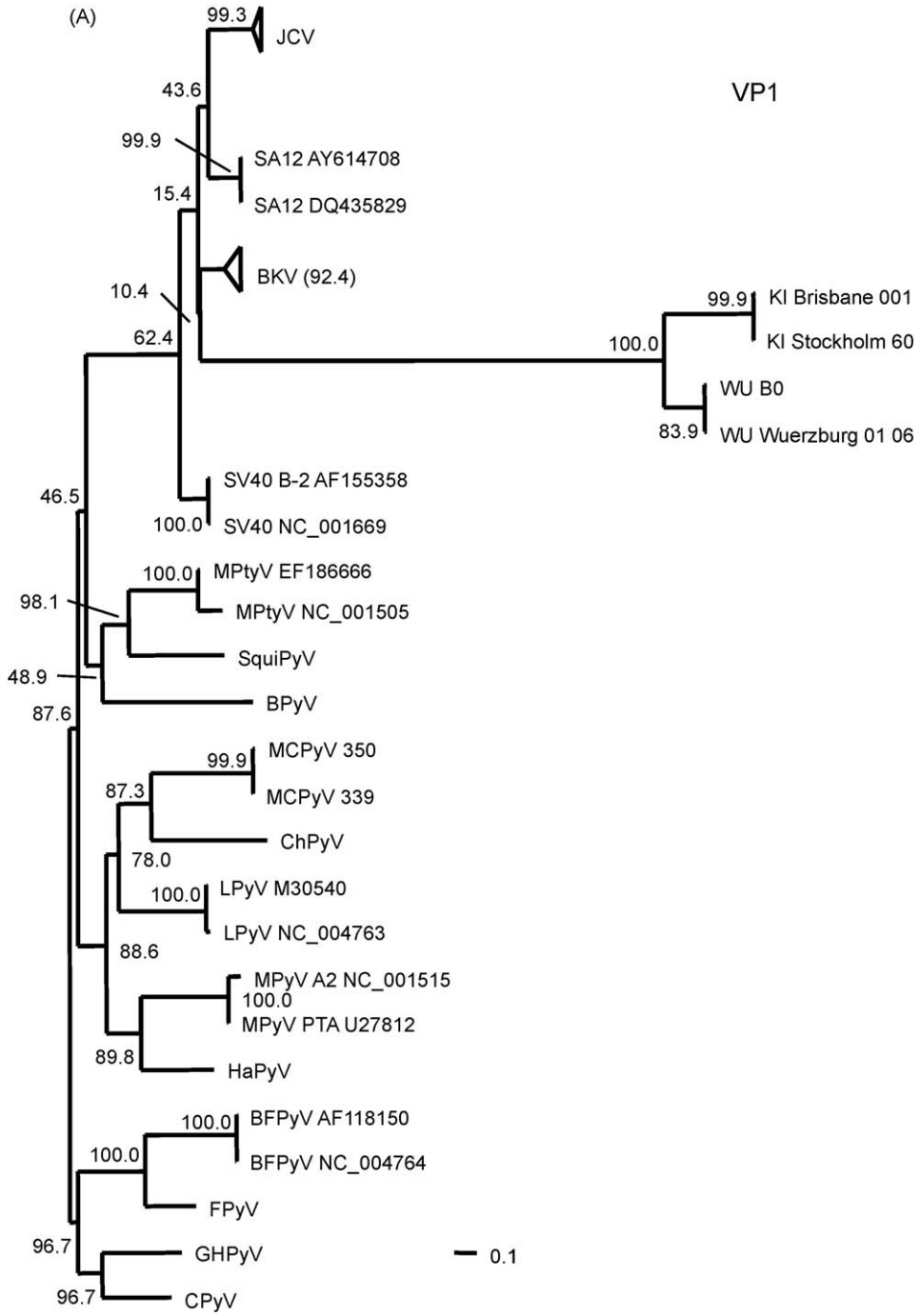

Fig. 3. Phylogenetic relationships of polyomaviruses. Maximum likelihood (ML) trees are given for VP1 (a), VP2 (b), T-ag (c), and for the concatenated data set of all three genes (d) (a brief description of the used method is given in supplementary material & methods section). BKV and JCV clades are condensed for the sake of clarity (for a detailed view, please refer to supplementary Fig. 1 in the supplementary material & methods section). Bootstrap values are indicated for the major nodes. MPtyV, the only mammalian polyomavirus species that can cause severe acute infections, does not cluster separately from other mammalian viruses. Note that the polyphyly of the human viruses WU/KI, JCV, BKV and MCPyV argue against a strict host-virus codivergence; other non-codivergence events are also apparent (see also Fig. 4). Compared to their branch lengths for T-ag, WU and KI are highly divergent for VP1 and VP2. This fact might reflect a faster evolution of WU and KI structural genes compared to that of other polyomaviruses. It additionally indicates that polyomaviral structural and non-structural genes can evolve with different rates. In all trees, branch lengths are proportional to genetic divergence. The scale bars indicate nucleotide substitutions per site.

contributions from the SV40 and SA12 sequences (results not shown).

Indeed, there appears to be reasonable evidence supporting that the emergence of BKV was linked to important steps in the evolution of modern humans (see also Zhong et al., 2009). When the timing of BKV evolution was calibrated against either of two "internal" events – the emergence of modern humans 200,000 years ago; out-of-Africa migration 100,000 years ago (the latter following previous works on JCV: Hatwell and Sharp, 2000; Sugimoto et al., 2002; Takasaka et al., 2006b) – the diversification of most BKV subgroups coincided with the radiation of modern humans less than 50,000 years ago (Krumbholz et al., 2008a). The dated tree also shows a reasonable fit to the observed geographical prevalences of BKV subtypes and groups (Krumbholz et al., 2008a). The inference of codivergence based on the congruence of the timing of evolutionary divergence events must always be viewed cautiously, however. This is especially true in the case of viruses, where the lack of a fossil record often necessitates the application of a molecular clock or selecting calibration points based on presumed codivergence events. For example, timing the BKV radiation based on the external calibration point tying the separation of human and simian polyomaviruses to the divergence of Old World monkeys (Cercopithecidae) and humans (Hominoidea) 23 million years

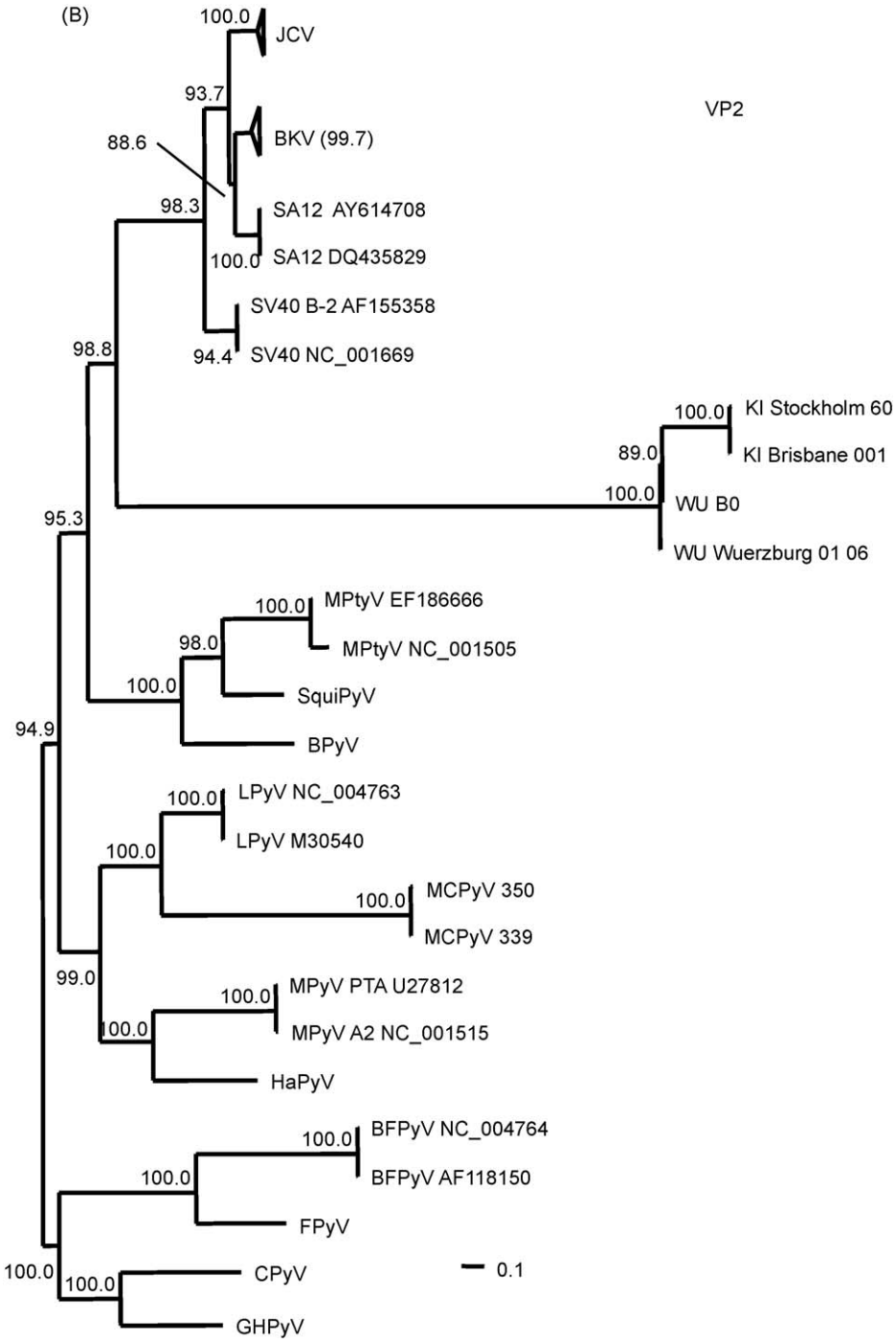

Fig. 3. (*Continued*)

ago results in the conclusion that BKV evolution was already completed by the time of appearance of modern humans 200,000 years ago (Krumbholz et al., 2008a; Nishimoto et al., 2006). Based upon our current results, however, this type of calibration point should now be viewed as being suspect because of the apparent lack of codivergence between polyomaviruses and their hosts at this level (as determined based solely on topological and not temporal congruence).

Important here is that codivergence is based primarily on topological congruence between host and virus phylogenies (as we have done) and less so on inferred divergence times given the extreme difficulty in inferring the latter in viruses. The problems inherent to external calibration points was mentioned in Krumbholz et al. (2008a). However, even internal calibration points can be problematic for inferring times of divergence because of the rate heterogeneity that is apparent between different polyomaviruses (e.g., the large divergence of WU and KI for the VP1 and VP2 genes; Fig. 3a and b) and even between different genes in the same species. For instance, it is clear that different BKV genes evolve at different rates (e.g., VP1 evolves faster than T-ag), with some even evolving in a clock-like fashion (Krumbholz et al., 2008a). Thus, timing events should ideally be based on multiple

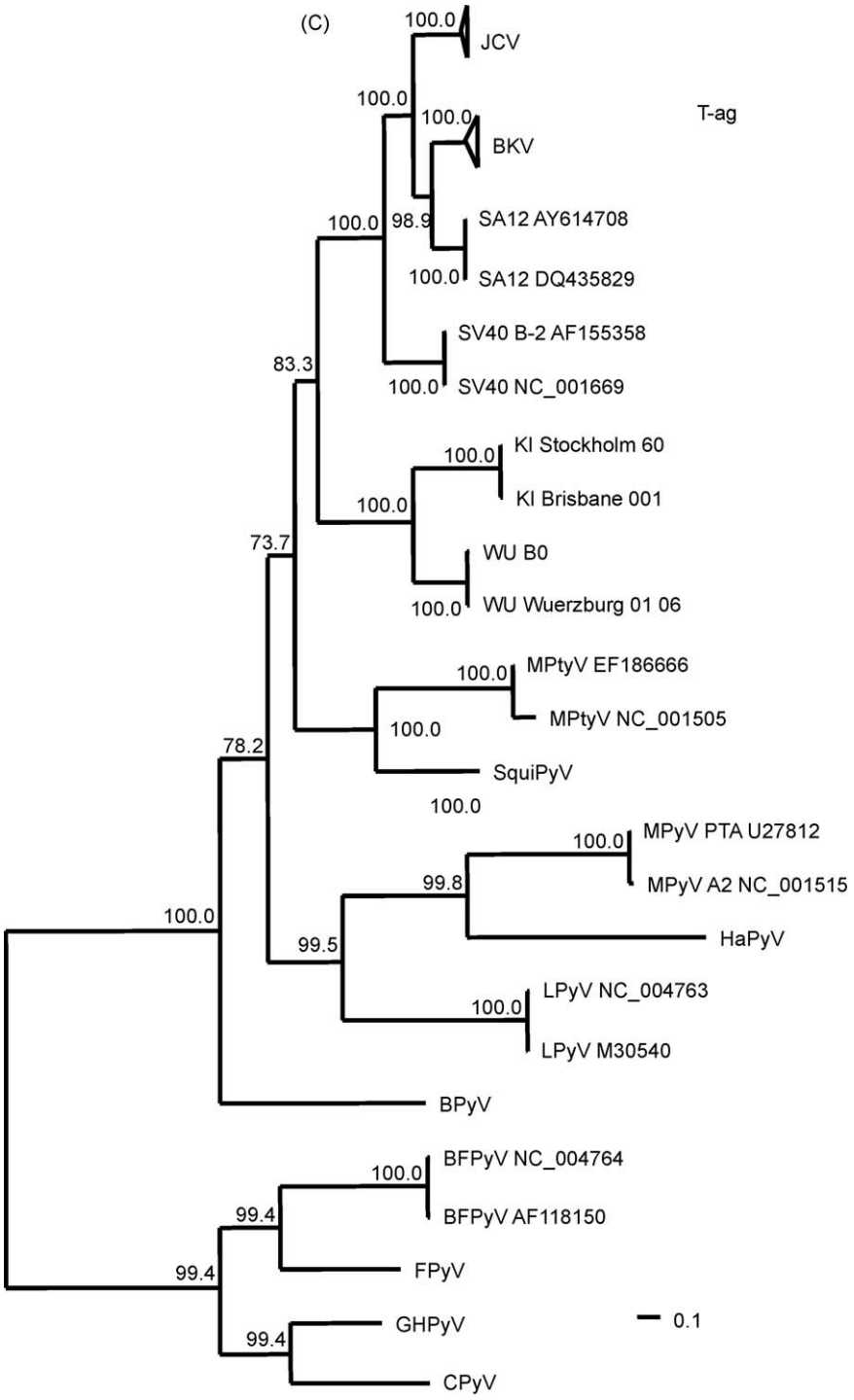

Fig. 3. (*Continued*)

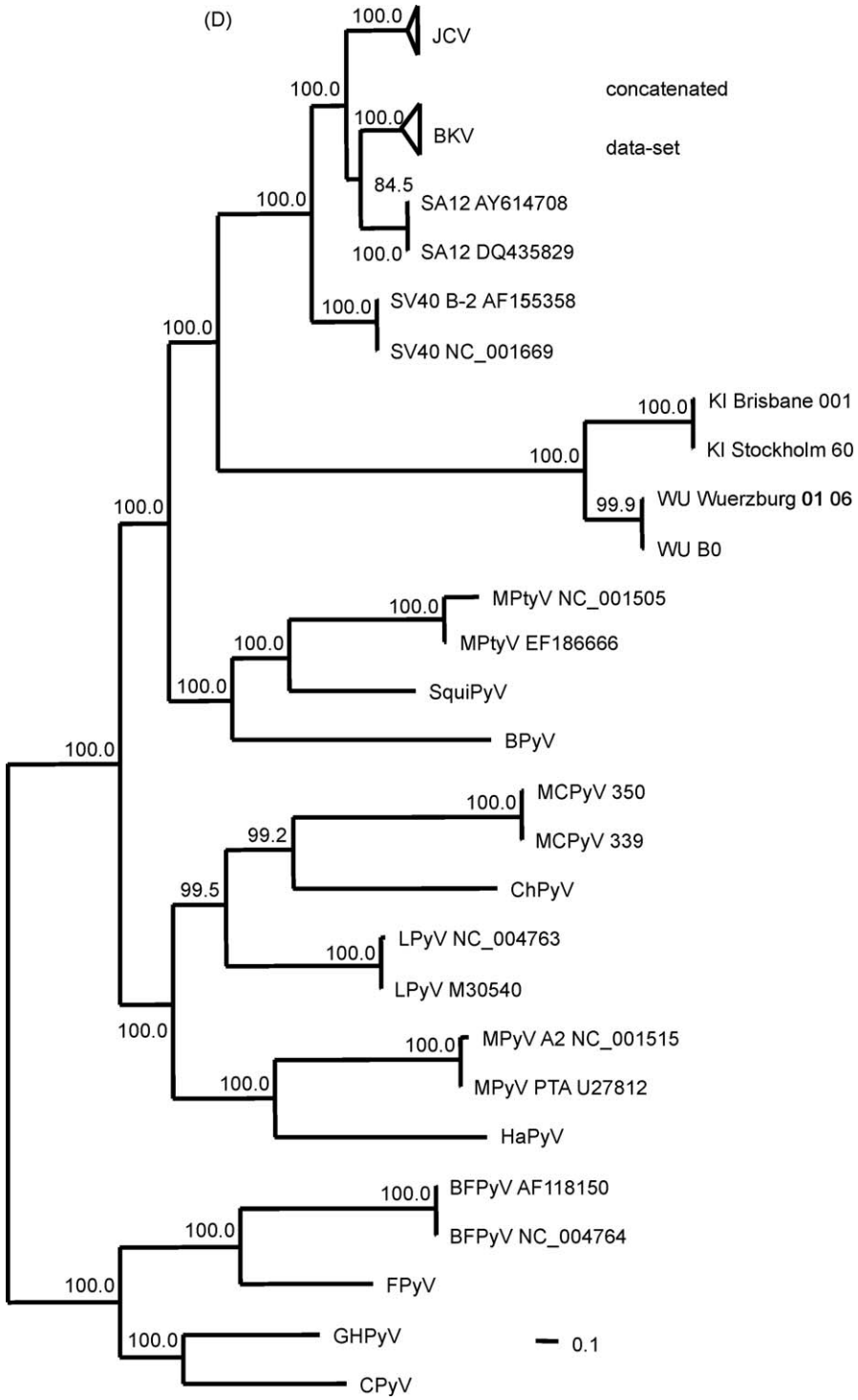

Fig. 3. (*Continued*).

independent estimates derived from different genes and the results examined critically.

Especially problematic appear to be estimates of evolutionary rates in human polyomaviruses (and by extension of divergence times) based on very recent (serial) human sampling, which yield values at least two order of magnitude faster than do other methods. For example, in attempting to estimate the intrahost substitution rate of BKV, Chen and colleagues observed up to four nucleotide substitutions of cloned BKV DNA that were obtained from one healthy and two immunocompromised individuals. Assuming that these substitutions have been accumulated over a period of 50 years, they calculated an evolution rate of (2–5) $\times 10^{-5}$ substitutions per site per year (Chen et al., 2004), implying that BKV diversification took place in less than 1000 years. The results, however, could not be confirmed by a later study (Takasaka et al., 2006a), and dual or multiple BKV infection of the respective individuals was not excluded. Similarly, Shackelton et al. (2006) compared 158 JCV sequences collected from different individuals over a timespan of 33 years to estimate a substitution rate of 1.7×10^{-5} substitutions per site per year. According to this

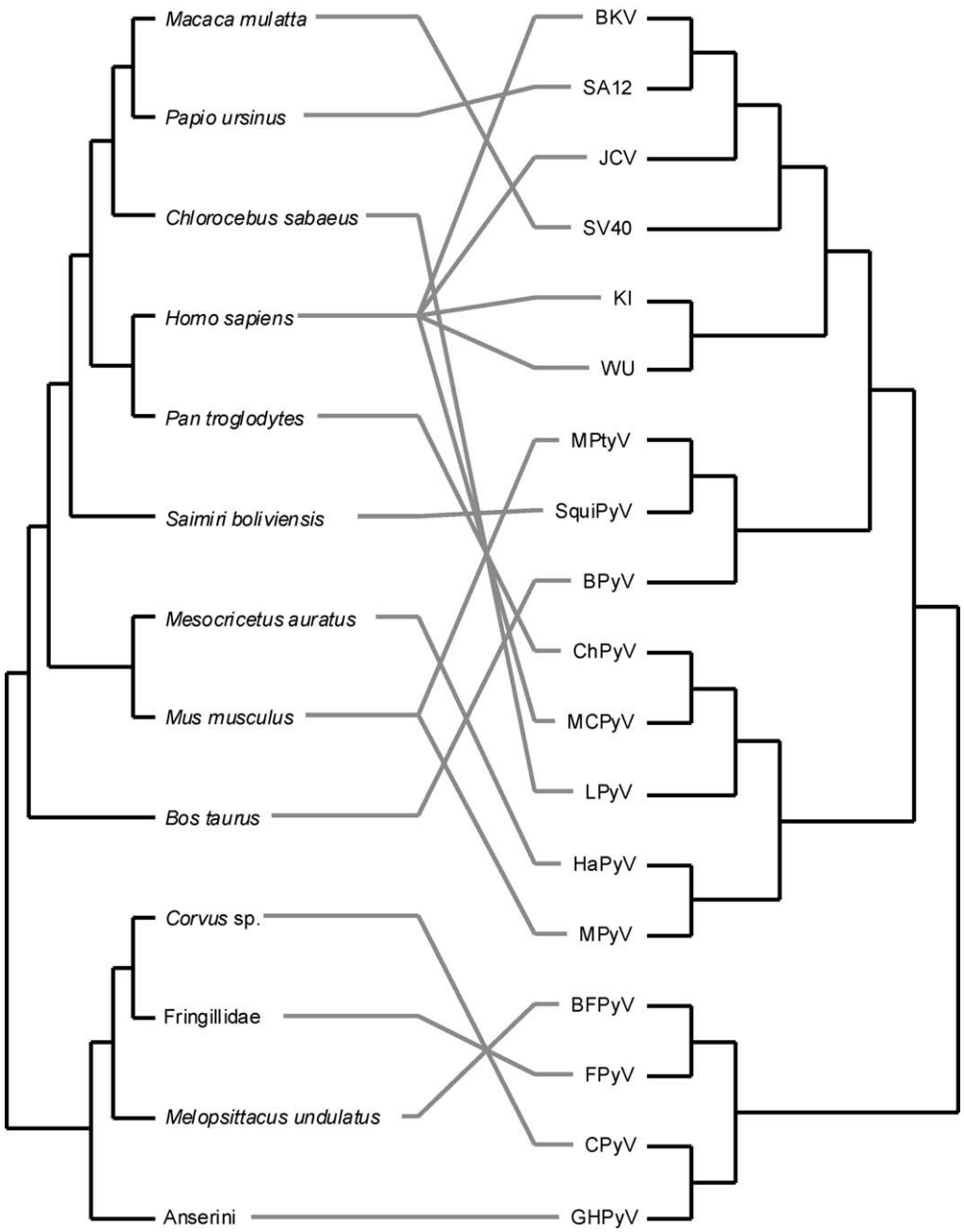

Fig. 4. Tanglegram showing phylogenetic relationships of each of the polyomavirus species and their hosts and the associations between the two. The polyomavirus phylogeny is a simplified version of that obtained from the analysis of the concatenated data set, restricted to the individual polyomavirus species. The host phylogeny is a composite based on a supertree of all extant mammalian species (Bininda-Emonds et al., 2007) and a recent review of bird phylogenetics (Cracraft et al., 2004). Crossing links between the viruses and their hosts indicate instances of non-codivergence, presumably due to host switching. A ParaFit analysis (Legendre et al., 2002) revealed that the degree of putative host switching unequivocally rejects the hypothesis of global codivergence between the viruses and their hosts (ParaFitGlobal = 114177153.0, $P = 0.49460$) and no individual links show significant codivergence (a brief explanation of the method is given in supplementary material & methods section, please further refer to supplementary Table 2 therein).

estimate, the JCV lineages would have evolved in the past 350 years.

Although serial sampling may be a suitable approach to estimate the substitution rate of a given genetic lineage, the data sets in these two studies are probably inappropriate with respect to their sampling strategy and restricted timespan of virus collection in the current context. Even in light of the apparent inverse correlation between mutation rate and genome size in dsDNA viruses (Duffy et al., 2008), such fast evolutionary rates (e.g., evolution of the numerous JCV lineages and BKV subgroups in only 10–30 human generations), even for the very small polyomaviruses, are astonishing given the more moderate evolutionary rates of the host-cell genome and the polymerases that mediate polyomavirus DNA replication in particular. Although viral replication rates and missing or altered mutation repair mechanisms may influence the fidelity of the replication process in infected cells, the sampling strategy with its inadequate sample size (see Shackelton et al., 2006), together with stochastic errors in rate estimation due to the small number of substitutions observed (and typically overestimates; see Duffy et al., 2008) and the potential for multiple infections of an individual may considerably influence such estimates.

Finally, all the approaches mentioned neglect the possible appearance of recombination and its adverse effects on the accurate estimation of the evolutionary relationships, genetic diversity and nucleotide substitution rates in polyomaviruses. To date, however, it remains controversial whether or not recombination occurs in polyomaviruses (Crandall et al., 2006; Hatwell and Sharp, 2000; Jobes et al., 1998).

6. Outlook

The past decade has seen an increasing interest in polyomaviruses, with many new species being discovered, if only by accident in some cases. To date, the most information is present for human and simian polyomaviruses, but the recent discoveries highlight both that a broader taxonomic range of hosts must exist and the possibility of additional human viruses (as well as multiple virus species for the other host species). Even for those viruses that are known, information about their clinical impact on their hosts (if any) is often lacking entirely. In reality, only the simian virus SV40 is comparatively well characterised.

Therefore, many questions about this family of double-stranded DNA viruses remain unanswered beyond the question of how widespread the group is in nature. For instance, it remains to be determined how typical the molecular biology of SV40 is for the entire group and, critically, what distinguishes persistent subclinical virus species from those presenting a pathological disease profile. Early suggestions and experimental evidence that lethality might be associated with a switch to a non-permissive host remain intriguing, but untested, especially in light of the lack of clinical information for most species and the lack of any significant global codivergence pattern between individual polyomavirus species and their hosts. Similar questions appear to be a hot topic currently in other virus groups including papillomaviruses (Gottschling et al., 2007), such that any answers might apply outside of any single group of viruses.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2009.04.008.

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