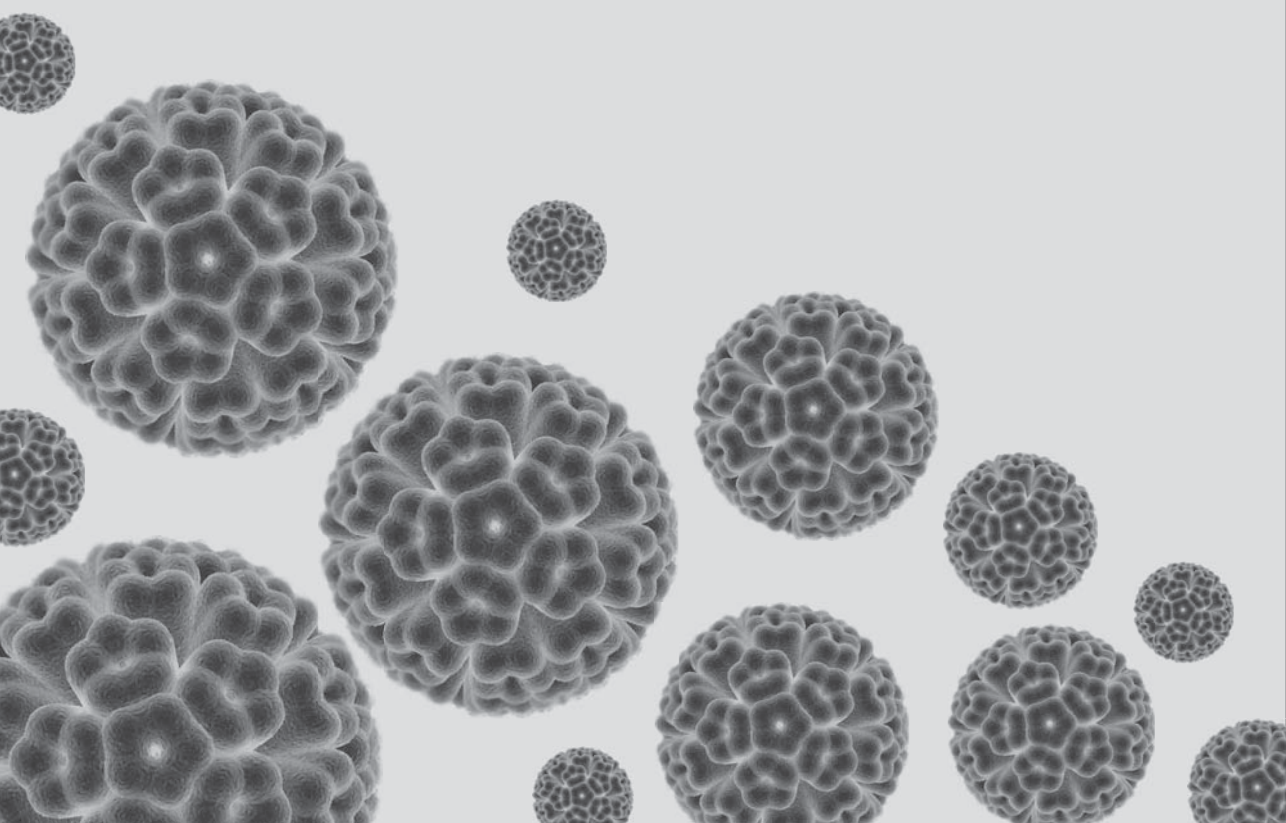

VePyV1 CaPyV JCPyV MPyV SA12 RacPyV GHPyV BKPyV
BatPyV CPyV OraPyV1 FPyV MptV APPyV1 SqPyV LPyV CSLPyV
EPyV PRPyV1 APPyV2 KIPyV MiPyV PtvPyV2c OtPyV1 STLPyV
MFPyV1 HPyV9 SV40 TSPyV CoPyV1 PPPyV CPPyV HPyV12
MXPyV PtvPyV1a PDPyV EiPyV1 AtPPyV1 HaPyV GggPyV1
CdPyV DRPyV MWPyV APyV CAPyV1 HPyV7 ChPyV MasPyV
WUPyV HPyV6 BPyV MCPyV OraPyV2 MMPyV SLPyV HPyV10

Part I

Introduction



Chapter 1

General introduction

Adapted from:*

From Stockholm to Malawi: recent developments in studying human polyomaviruses

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** Note: Adaptation of this chapter from the original published article concerns textual and figure adjustments. The text has been updated with novel data from the literature until mid-2014. Figures 2, 7 and 8 are updated with novel polyomaviruses. Figures 3, 5 and 6 were not part of the original publication.*

Abstract

Until a few years ago, the polyomavirus family (*Polyomaviridae*) included only a dozen viruses mostly identified in avian and mammalian hosts. Two of these, the JC and BK-polyomaviruses isolated a long time ago, are known to infect humans and cause severe illness in immunocompromised hosts. Since 2007, tens of new polyomaviruses were identified, including at least eleven that infect humans. Among them is the polyomavirus associated with trichodysplasia spinulosa (TSPyV). In this introductory chapter, the recent developments in studying the novel human polyomaviruses until mid-2014 are summarized, which sets the stage for further investigation into TSPyV infection, pathogenesis, evolution and host adaptation.

Historical background

Polyomaviruses have been recognized as a separate virus family (*Polyomaviridae*) since 1999. Before that time, they formed the genus Polyomavirus in the *Papovaviridae* family that also contained the genus Papillomavirus [1, 2]. The first members of the polyomavirus family, mouse polyomavirus (MPyV) and SV40, were identified halfway through the last century as filterable agents that caused tumors in newborn mice and hamsters [3 - 5]. Subsequently, the first two human polyomaviruses were discovered in 1971. The JC-polyomavirus (JCPyV) was identified in a brain tissue extract from a progressive multifocal leukoencephalopathy (PML) patient with the initials J.C. [6]. The first reported BK-polyomavirus (BKPyV) was isolated from the urine of a nephropathic kidney transplant patient with the initials B.K. [7]. Both JCPyV and BKPyV have no oncogenic capability in humans despite being phylogenetically closely related to SV40 that was associated with oncogenic properties in humans, but this issue remained controversial so far (**Figure 1**) [2, 8].

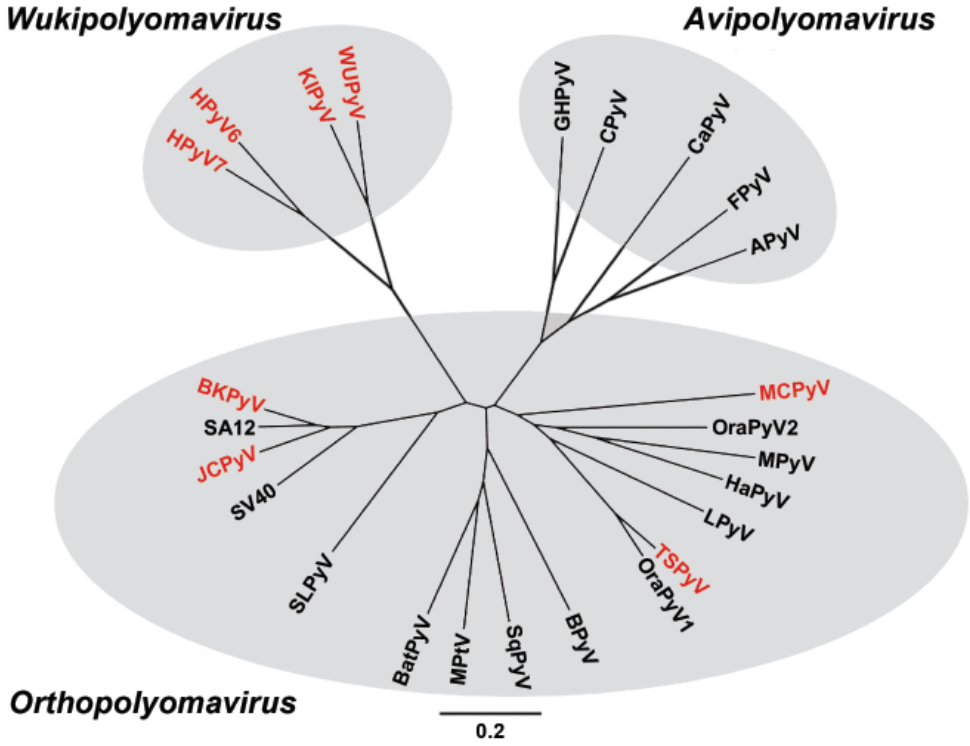

Figure 1. Phylogenetic grouping of 25 polyomaviruses that were known up until October 2010, including 8 human viruses (red), as proposed to the International Committee on Taxonomy of Viruses (ICTV). The tree is based on the whole genomic nucleotide sequences. Figure adapted from [2].

Subsequently, more polyomaviruses were identified in rodents, non-human primates, cattle and birds, but not in humans until this century. From 2007 on, at least eleven additional human polyomaviruses (HPyVs) were discovered. For three of those (i.e., MCPyV, TSPyV and NJPyV) involvement in disease development in immunosuppressed and/or elderly patients seems highly likely [9 - 11]. They seem to fit the 'opportunistic' polyomavirus profile already known for JCPyV and BKPyV characterized by symptomatic reactivation after a long period of persistent latent infection. For the other novel HPyVs, disease association has not been demonstrated and their pathogenicity is still unknown.

A timeline of a selection of polyomavirus discoveries is illustrated in **Figure 2**. A list of all polyomaviruses identified up until mid-2014, which prototype the (tentative) namesake species are shown in **Table 1**, including name abbreviations and GenBank (RefSeq) numbers of genome sequences.

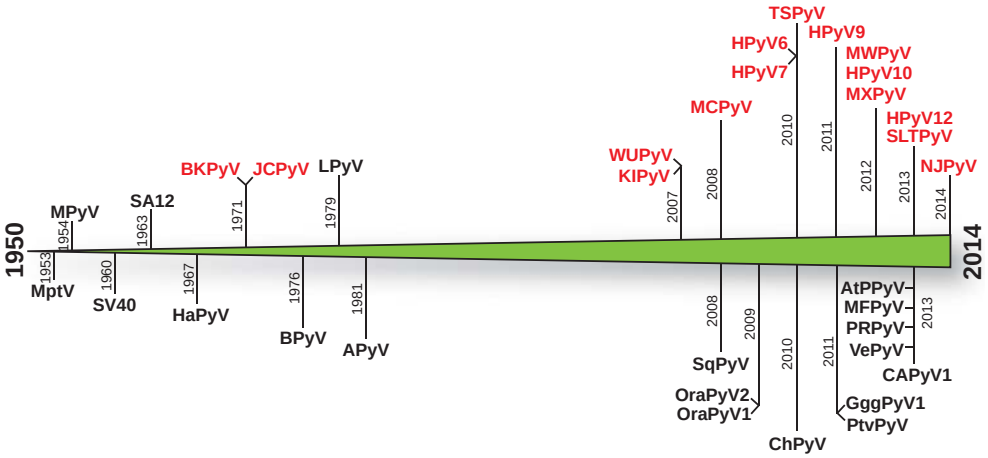

Figure 2. A time line is shown that indicates the discovery of mainly human (red) and a number of animal polyomaviruses until June-2014. Additionally discovered mammal and bird polyomaviruses are indicated in **Table 1**. The year of discovery refers to the first description of the virus, not necessarily to the year that the full DNA genome was sequenced. The abbreviated names of the viruses are explained in **Table 1**.

Identification of new human polyomaviruses

With the development of nucleic acid amplification and detection techniques, the need for cytopathic changes observed in cell culture to detect the presence of a virus was bypassed. Recent versions of these molecular techniques allowed sensitive and high-throughput analyses of large number of clinical samples that led to a revolution of virus discovery. These techniques are frequently combined with strategies to enrich the original sample for viral DNA or RNA by reducing the content of hosts genomic DNA. Here, recent discoveries of human polyomaviruses are summarized.

Table 1: List of 58 polyomavirus names identified up to mid-2014

Host type	Polyomaviruses	Year*	RefSeq**
Human	BK polyomavirus (BKPyV)	1971 [6]	NC_001538
	JC polyomavirus (JCPyV)	1971 [7]	NC_001699
	KI polyomavirus (KIPyV)	2007 [12]	NC_009238
	WU Polyomavirus (WUPyV)	2007 [13]	NC_009539
	Merkel cell polyomavirus (MCPyV)	2008 [9]	NC_010277
	Human polyomavirus 6 (HPyV6)	2010 [10]	NC_014406
	Human polyomavirus 7 (HPyV7)	2010 [14]	NC_014407
	Trichodysplasia spinulosa-associated polyomavirus (TSPyV)	2010 [14]	NC_014361
	Human polyomavirus type 9 (HPyV9)	2011 [15]	NC_015150
	Malawi polyomavirus (MWPyV)	2012 [16]	NC_018102
	Human polyomavirus type 10 (HPyV10)	2012 [17]	JX262162
	Mexico polyomavirus (MXPyV)	2012 [18]	JX259273
	St. Louis polyomavirus (STLPyV)	2013 [19]	NC_020106
	Human polyomavirus type 12 (HPyV12)	2013 [20]	NC_020890
	New Jersey polyomavirus (NJPyV)	2014 [11]	KF954417
Non-human primate	Simian virus 40 (SV40)	1962 [3]	NC_001669
	Baboon polyomavirus 1 (SA12)	1963 [21]	NC_007611
	B-lymphotropic polyomavirus (LPyV)	1979 [22]	NC_004763
	Squirrel monkey polyomavirus (SqPyV)	2008 [23]	NC_009951
	Bornean orang-utan polyomavirus (OraPyV1)	2010 [24]	NC_013439
	Sumatran orang-utan polyomavirus (OraPyV2)	2010 [24]	FN356901
	Chimpanzee polyomavirus (ChPyV)	2010 [25]	NC_014743
	Gorilla gorilla gorilla polyomavirus (GggPyV1)	2011 [26]	HQ385752
	Pan troglodytes verus polyomavirus (PtvPyV1a)	2011 [26]	HQ385746
	Pan troglodytes verus polyomavirus (PtvPyV2c)	2011 [26]	HQ385749
	Cebus albifrons polyomavirus (CAPyV1)	2013 [27]	NC_019854
	Ateles paniscus polyomavirus (AtPPyV1)	2013 [27]	NC_019853
	Macaca fascicularis polyomavirus (MFPyV1)	2013 [27]	NC_019851
	Piliocolobus rufomitratus polyomavirus (PRPyV1)	2013 [27]	NC_019850
	Vervet monkey polyomavirus (VePyV1)	2013 [27]	NC_019844
Mammal (other)	Murine pneumotropic virus (MptV)	1953 [28]	NC_001505
	Murine polyomavirus (MPyV)	1953 [5]	NC_001515
	Hamster polyomavirus (HaPyV)	1967 [29]	NC_001663
	Bovine polyomavirus (BPyV)	1976 [30]	NC_001442
	Bat polyomavirus (BatPyV)	2009 [31]	NC_011310
	California sea lion polyomavirus (CSLPyV)	2010 [32]	NC_013796
	Mastomys polyomavirus (MasPyV)	2011 [33]	AB588640
	Equine polyomavirus (EPyV)	2012 [34]	JQ412134
	Molossus molossus polyomavirus (MMPyV)	2012 [35]	JQ958893
	Desmodus rotundus polyomavirus (DRPyV)	2012 [35]	JQ958892
	Pteronotus parnellii polyomavirus (PPPyV)	2012 [35]	JQ958891
	Pteronotus davi polyomavirus (PDPyV)	2012 [36]	NC_020070
	Artibeus planirostris polyomavirus (APPyV2)	2012 [35]	JQ958890
	Artibeus planirostris polyomavirus (APPyV1)	2012 [35]	JQ958887
	Carollia perspicillata polyomavirus (CPPyV)	2012 [35]	JQ958889
	Sturnira lilium polyomavirus (SLPyV)	2012 [35]	JQ958888
	Chaerephon polyomavirus (CoPyV1)	2012 [36]	NC_020065
	Cardioderma polyomavirus (CdPyV)	2012 [36]	NC_020067
	Eidolon polyomavirus (EiPyV1)	2012 [36]	NC_020068
	Miniopterus polyomavirus (MiPyV)	2012 [36]	NC_020069
	Otomops polyomavirus (OtPyV1)	2012 [36]	NC_020071
	Raccoon polyomavirus (RacPyV)	2013 [37]	JQ178241
	African elephant polyomavirus (AelPyV)	2013 [38]	NC_022519
Bird	Avian polyomavirus (APyV)	1981 [39]	NC_004764
	Goose hemorrhagic polyomavirus (GHPyV)	2000 [40]	NC_004800
	Finch polyomavirus (FPyV)	2006 [41]	NC_007923
	Crow polyomavirus (CPyV)	2006 [41]	NC_007922
	Canary polyomavirus isolate (CaPyV)	2010 [42]	GU345044

*** Year of first description, not necessarily the year of first complete genome sequencing**

**** Genome sequence according GenBank (RefSeq) number (http://www.ncbi.nlm.nih.gov/RefSeq/)**

KI-polyomavirus (KIPyV) and WU-polyomavirus (WUPyV)

In 2007, almost coincidentally, two novel HPyVs were reported; the Karolinska Institute polyomavirus (KIPyV) identified in Stockholm (Sweden), and the Washington University polyomavirus (WUPyV) identified in St. Louis (USA). Both were discovered in respiratory tract samples from individuals with (acute) respiratory tract infections, but by different methods.

KIPyV was identified by pooling centrifuged and filtered supernatants from randomly selected DNase-treated nasopharyngeal aspirates, from which DNA and RNA were extracted [12]. The nucleic acids served as a template for random-PCR, and the products were separated by gel electrophoresis, cloned and sequenced. This resulted in several sequence reads that were subsequently trimmed, clustered and sorted using dedicated software. Finally, BLAST searches were performed to look for similarities with known (viral) sequences listed in GenBank.

For the identification of WUPyV, a 'shotgun sequencing' strategy was used on a single nasopharyngeal aspirate that proved negative for 17 known viral respiratory pathogens in diagnostic PCR. Total nucleic acid isolated from the sample was randomly amplified and PCR products were cloned and sequenced. The DNA sequence reads were analyzed in a comparable fashion to the above for KIPyV, which revealed several sequences that displayed significant homology with JCPyV and BKPyV [13]. Subsequent analyses of the compiled WUPyV genome showed highest sequence homology to KIPyV.

Merkel cell polyomavirus (MCPyV)

The discovery of MCPyV proved the assumptions of polyomavirus involvement in human oncogenesis finally to be true. Merkel cell carcinomas (MCC) are a rare but aggressive type of cutaneous tumors of neuroendocrine origin that typically develop in the elderly and immunocompromized patients (see paragraph "The new human polyomaviruses and their associated diseases"). Using a technique called the digital transcriptome subtraction [43], MCPyV was identified in 8 out of 10 analyzed MCC lesions [9]. This technique compares (subtracts) cDNA libraries generated with random RT-PCR from diseased and healthy tissues and thus can identify messengerRNA transcripts potentially unique for either or both. In this case, with the help of sophisticated software, unique and unknown sequences were followed-up, of which some displayed homology to the monkey B-lymphotrophic polyomavirus (LPyV) and BKPyV. Through primer-genome-walking, subsequently the complete genome was sequenced. New to HPyVs, but known for oncogenic animal polyomaviruses detected in tumors, the MCPyV genome was found to be monoclonally randomly integrated into the genome of the analyzed MCC lesions [9, 44].

Human polyomavirus 6 and 7 (HPyV6 and HPyV7)

In order to detect free, non-integrated, MCPyV genomes on the skin, rolling-circle amplification (RCA) was performed on forehead swab samples of healthy individuals. Performing the RCA technique on samples results into amplification of circular double-stranded viral DNA

by using random primers and strand-displacement DNA polymerase (**Figure 3**) [45]. With some adjustments to boost the RCA sensitivity, this approach revealed the identity of a new polyomavirus, called the type 6 (HPyV6), on the skin of a healthy individual [14]. By using a newly developed degenerate PCR primer-set on the similar pre-treated DNA materials from those healthy skin samples to detect HPyV6- and WUPyV-like sequences, an additional new polyomavirus sequence was identified and completely sequenced, which was subsequently named HPyV7 [14].

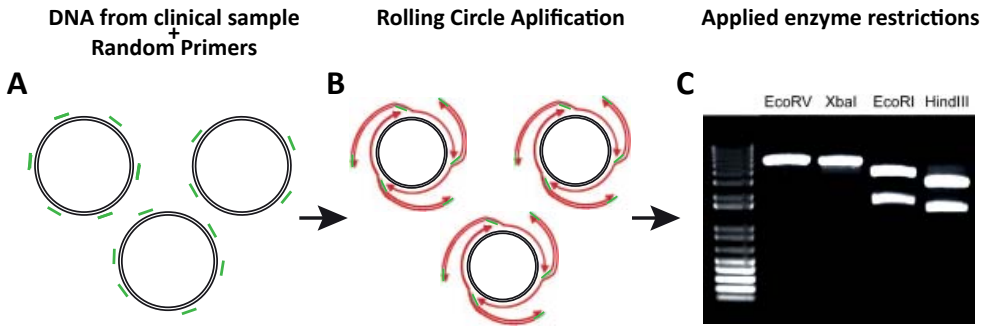

Figure 3. The rolling-circle amplification (RCA) technique exploits random hexamer primers and a DNA polymerase (Phi-29) with proofreading and strand-displacement capabilities used on clinical DNA samples (**A**). If a sample contains a relative excess of a particular small circular double-stranded DNA molecule, for instance a plasmid or a (small) viral genome, RCA will produce long, high-molecular DNA molecules with concatenated (linearized) stretches of DNA of potential interest (**B**). These stretches will appear as sharp bands on gel when the RCA-product is cut with specific restriction enzymes that can subsequently be cloned and sequenced (**C**). Figure adapted from [10, 45].

Trichodysplasia spinulosa-associated polyomavirus (TSPyV)

Trichodysplasia spinulosa (TS) is a rare skin disease of immunocompromized patients, which will be described in more detail below and in **Chapter 2**. Already in 1999, Haycox and co-workers proposed that TS has a viral origin, because TS lesions contained clusters of small virus particles [46]. It was only in 2010 that the identity of the virus was revealed [10], with the help of RCA technique (**Figure 3**) [45]. With specific primers used during primer-genome-walking the entire RCA product was sequenced, which revealed the entire TSPyV genome in lesions of a TS patient [10].

Human polyomavirus 9 (HPyV9)

Previously, several serological studies suggested the presence of 'cross-reactive' antibodies in a percentage of human sera that recognized a non-human primate polyomavirus (LPyV isolated from an African green monkey) capsid-protein [47 - 49]. Furthermore, LPyV-derived sequences were obtained from human blood, indicating the occurrence of LPyV(-like) infections in humans [50]. In 2011, a novel polyomavirus that closely resembled LPyV was identified, independently, by two research groups in human serum and skin swabs, respectively,

and tentatively called HPyV9. One group used a degenerate polyomavirus PCR primer-set [26] to screen serum samples, which generated an unknown, putative HPyV sequence, next to some known HPyV sequences. With primer-genome-walking, the rest of the HPyV9 genome was sequenced [15]. The other group isolated DNA from healthy skin swabs and subjected the DNA to high-throughput sequencing, which resulted in millions of reads and several contigs that matched MCPyV, HPyV6 and HPyV7, as well as many human papillomaviruses. Some of the contigs showed the highest homology to LPyV and after filling the contig sequence gaps by designing additional primers, HPyV9 genome was identified [51].

Malawi, Mexico and human polyomavirus 10 (MWPyV/MXPyV/HPyV10)

Another novel human polyomavirus was identified in stool of a child from Malawi [16]. This virus was discovered by suspending a small amount of feces that subsequently was centrifuged and filtered through 0.45 mm and then 0.22 mm pores. After chloroform and DNase treatment, extraction-free-DNA was obtained. The solution that still contained encapsidated DNA was SDS and Proteinase K-treated, and DNA was isolated. Finally, this DNA was subjected to RCA and pyrosequencing. Hundreds of reads were obtained, sequenced and aligned to sequences from the GenBank database. This revealed several polyomavirus-like reads and with the use of primer-genome-walking strategy the complete MWPyV genome sequence was fulfilled [16].

Shortly after, two almost identical viruses were identified, called the HPyV10 and Mexico polyomavirus (MXPyV). HPyV10 genome was isolated from an anal wart of an immunocompromized patient with WHIM syndrome with the help of RCA and primer-genome-walking sequencing [17]. MXPyV was identified in diarrheal stool collected from a child in Mexico [18]. From stool, viral particles were purified in a PBS suspension, containing glass beads and chloroform, by mechanical shaking and centrifugation. Further processing of the filtered supernatant was similar as for MWPyV genome identification described above. Sequencing of the barcoded reads was performed with an Illumina HiSeq 2000. Assembled contigs from 75bp reads were used to design primers directed outward for use in long-range PCR to amplify and sequence the whole genome. Because MWPyV, HPyV10 and MXPyV share at least 95% of their genome sequence, they most probably represent the same polyomavirus species.

St. Louis polyomavirus (STLPyV)

As part of a human gut-microbiome-survey study of healthy and malnourished children from Malawi performed by the same group that identified the WUPyV and MWPyV, another new polyomavirus was discovered and called STLPyV [19]. Prior to DNA extraction, the stool was processed by CsCl ultracentrifugation to generically enrich for viral particles. After DNA was extracted and subsequently amplified by a technique called multiple displacement amplification (MDA), shotgun 454 pyrosequencing was performed. MDA technique is comparable to the RCA method exploiting random hexamer primers and a DNA polymerase (Phi-29) with

proofreading and strand-displacement capabilities (**Figure 3**). After sequencing, two reads were identified in this sample, which shared limited sequence identity to known polyomaviruses. Employing these two initial reads, primers were designed to PCR amplify products that span the whole genome in either direction. STLPyV has a mosaic genome that probably shares an ancestral recombinant origin with the MWPyV because of its 64.2% genome similarity to this polyomavirus.

Human polyomavirus 12 (HPyV12)

Very recently, a twelfth human polyomavirus was isolated (HPyV12) [20]. HPyV12 genome was tested positive in 11% of liver tissues analyzed in that study. Methods used for genome identification and sequencing were identical/comparable to the identification of HPyV9 [15]. HPyV12 is distantly related to other viruses from the *Orthopolyomavirus-I* genogroup (see paragraph "Phylogeny and evolutionary trends"). Whether this virus is involved in pathogenesis of a liver disease, or other gastrointestinal diseases, remains to be seen.

New Jersey polyomavirus (NJPyV)

The most recently identified human polyomavirus, called the New Jersey polyomavirus (NJPyV), was isolated from an immunosuppressed patient diagnosed with vasculitic myopathy and retinopathy caused by microthrombosis. In tested biopsies, clusters of viral particles were observed in swollen nuclei of endothelial cells [11]. The authors identified the viral genome after extraction of RNA from sections of formalin-fixed paraffin-embedded muscle tissue, from which they generate cDNA libraries that were subsequently used for sequencing. The obtained sequence reads were all uninformative by nucleotide sequence analysis (BLASTn), but when translated amino acid analysis (BLASTx) was performed, they found approximately 80% sequence homology with chimpanzee polyomaviruses. Future studies should investigate whether this virus has indeed a tropism for vascular endothelial cells and may be involved in vasculitis in general. Interestingly, the patient had to evacuate through floodwaters because of superstorm Sandy weeks prior to development of her symptoms, prompting speculation about NJPyV contagion through sewage-contaminated waters.

Altogether, since 2007 an explosion in human polyomavirus discoveries took place, among which the discovery of TSPyV is evident. These discoveries are most likely due to improvement in viral DNA purification and amplification methods and particularly the use of high-throughput Next Generation Sequencing to identify viral DNA with low copy-numbers in the background of excess host genomic DNA. Identification of additional viruses of the *Polyomaviridae* family and studies of their genome, epidemiology, pathogenicity and evolution will undoubtedly lead to a better understanding of the role these viruses play in human health and disease.

The polyomavirus genome and its products

Genome organization and transcription

Viruses of the *Polyomaviridae* family, including the recently identified human viruses, are among the smallest known to infect humans, both in particle size and in genome length [52]. The non-enveloped virions of about 45-nm harbour a circular double-stranded DNA genome of approximately 5000 basepairs that is divided into three regions; (i) the non-coding control region (NCCR) and in opposing directions, (ii) the early (T-antigen) and (iii) the late (VP) coding regions (**Figure 4**).

The NCCR contains the origin of replication flanked by several large T-antigen-binding sites, transcription promoters and regulatory elements. The novel human polyomaviruses display a similar NCCR organization. In JCPyV- or BKPyV-diseased individuals, the NCCR of virus isolates found in different body compartments can vary from what is called the 'archetype' NCCR that is present in transmissible virus found in urine for example. It is believed that the rearranged NCCR increases the T-antigen transcription and replication rate, as was shown for instance for JCPyV [53]. Systematic comparisons of NCCR regions among different isolates found within diseased and healthy individuals have not been reported (yet) for the novel human polyomaviruses.

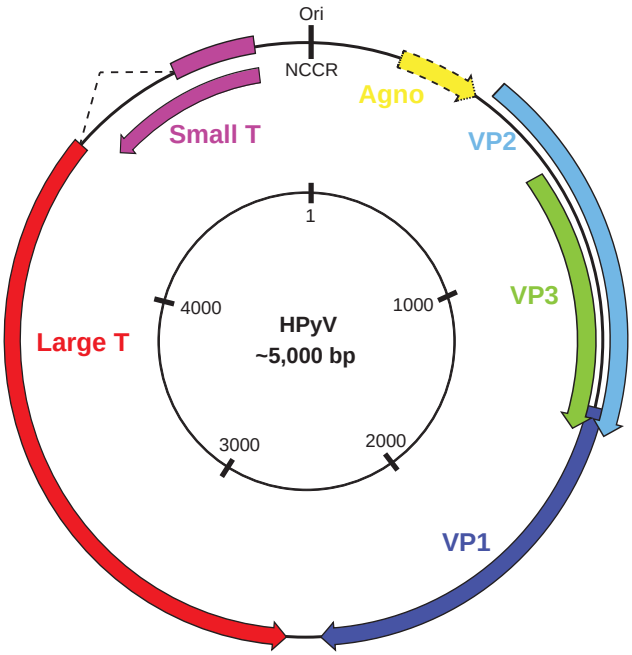

Figure 4. Old schematic representation of human polyomavirus circular double-stranded DNA genome organization as proposed before, showing only the protein-expressing layer. Updated representations, that include middle T-antigen and ALTO protein, are shown in **Chapter 2** and **Chapter 6**. Regions coding for the indicated proteins are depicted in different colors. The agnoprotein (yellow) is encoded only by some HPyVs.

The T-antigen coding region of most HPyVs encodes at least the small and large T-antigens that are translated from an alternatively spliced pre-messengerRNA. In addition, shorter or alternatively spliced products of T-antigens are expressed, such as 17kT, T4 and 57kT [54, 55]. Rodent polyomaviruses are known to express an additional T-antigen, called middle T-antigen [56]. The VP coding region found on the opposite strand codes for at least the VP1 and VP2 structural capsid proteins, and often VP3. The agnoprotein and VP4 expressed by JCPyV, BKPyV and several animal polyomaviruses, including birds, seem not to be encoded by the newly identified HPyVs [57], but for most of these viruses the transcription-patterns have not been solved yet.

Besides the NCCR transcription regulation, for example shown for SV40, T-antigen messengerRNAs are post-transcriptionally regulated by microRNAs [58]. In addition to SV40, also for MCPyV and BKPyV a microRNA was identified that potentially regulates early gene expression [59, 60].

T-antigens

Polyomavirus T-antigens play essential roles in viral transcription and replication, as well as in host-cell tuning to enable efficient virus replication [52, 61]. As polyomaviruses are completely dependent on the host cell replication machinery, a significant part of this tuning is aimed at inducing S-phase and bypassing cell cycle-control measures. In some cases, these features can derail and result in uncontrolled cell proliferation and eventually tumor formation. So far, MCPyV is the only (newly identified) HPyV for which these phenomena have been noted [62]. Consequently, the T-antigens of MCPyV have been studied already quite extensively (see next, and “MCPyV and MCC” paragraph), whereas for the other new HPyVs this knowledge is almost entirely based on predicted motifs in protein sequences.

The small and large T-antigens share their N-terminus, while having C-terminal regions of different sizes encoded from different reading frames (**Figure 4**). The novel human polyomaviruses seem no exception to this rule. This shared region occupied by the so-called J-domain contains important motifs, such as CR1 and DnaJ, for viral replication and cellular transformation [61]. Downstream, the small T-antigen contains a PP2A subunit-binding motif probably involved in activation of the Akt-mTOR pathway related to (tumor) cell survival [63, 64]. The part of large T-antigen, encoded by the second exon, includes many functionally important domains or motifs such as: (i) a nuclear-localization signal, (ii) an origin-binding domain, (iii) an ATPase-containing helicase domain [61, 65], (iv) a pRB-binding and (v) sometimes a p53-interaction domain that both potentially play a crucial role in cellular transformation. Apart from MCPyV [55], the presence of (most of) these motifs and domains was identified only by bioinformatics approach in TSPyV and HPyV9 [10, 15], but they remain to be characterized functionally.

Murine (MPyV) and hamster (HaPyV) polyomaviruses express also a middle T-antigen, which serves as the prime oncoprotein during cellular transformation [56]. Middle T-antigen has a C-terminal transmembrane domain, several Tyr, Ser and Thr phosphoryla-

tion sites, and some other functionally unknown conserved domains. Phosphorylation of the Tyr, Ser and Thr residues of middle T-antigen results into binding and activation of several cellular signal transduction proteins (e.g., Src, PI3K, PLC-gamma1) that promotes cellular transformation and oncogenesis [56, 66 - 68].

VP capsid proteins

Together, SV40 encoded late VP structural proteins make up the approximately 45-nm polyomavirus capsid [69]. VP1 molecules interact with VP2 or VP3 molecules to auto-assemble into a full capsid-complex (**Figure 5**) [52]. Comparable VP1, VP2 and/or VP3 encoding sequences have been suggested for the novel polyomaviruses. So far, there is no reason to believe that the novel viruses will behave differently with respect to capsid formation, although there is debate whether MCPyV VP3 is expressed or is functional [70, 71]. The VP4 protein, noted for SV40, is expressed very late in infection and serves probably as a porin of cellular membrane that facilitates the viral release [72, 73]. So far, this protein has not been predicted or reported for any of the newly identified HPyVs.

How the polyomaviruses recognize and infect their host target cells is partly known. Most of the known polyomaviruses interact by linkage of the major capsid protein VP1 with terminal sialic acids (alpha2,3 or alpha2,6) of mainly gangliosides on the host cell membrane [74], after which they are internalized using different entry pathways. For MCPyV, it was shown that this virus uses glycosaminoglycans for the initial attachment, while the sialic acids may be important later in the encounter [75]. Future research could reveal whether the other novel human polyomaviruses use similar strategies for host cell recognition.

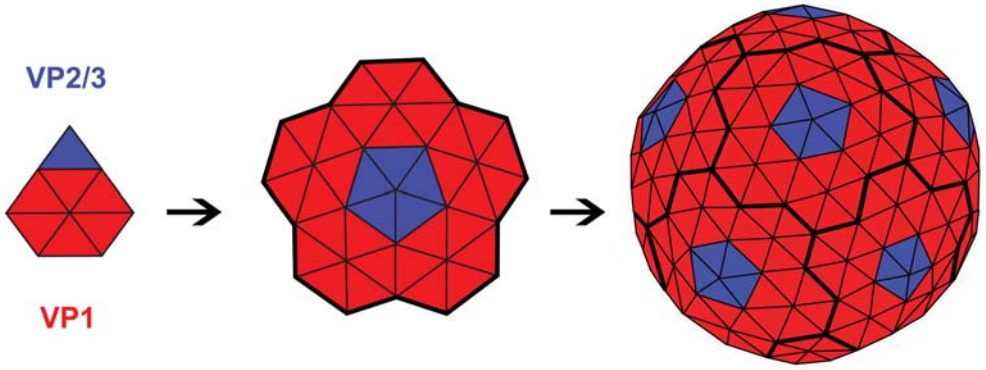

Figure 5. Illustration of polyomavirus icosahedral capsid formation. Six VP1 proteins interact with either a VP2 or VP3 to assemble into a hexameric protomere. Five of these protomeres interact to form a capsomere, which subsequently auto-assemble into a full capsid-complex [76].

The new human polyomaviruses and their associated diseases

For two of the novel HPyVs a causal relationship with a specific clinical condition is highly probable. In both cases, the disease/cancer has lent its name to the virus, trichodysplasia spinulosa to TSPyV and Merkel cell carcinoma to MCPyV. For MCPyV, an additional association with chronic lymphocytic leukemia that originates from B-cells was suggested, and for TSPyV an association with pilomatricoma that represents a benign hair follicular skin tumor was speculated. However, evidence for these correlations is (yet) weak. For the recently identified NJPyV associated with the vasculatory myositis, retinitis and dermatitis, the evidence is highly suggestive, but limited to one patient observation so far. For the other new human polyomaviruses, the evidence for involvement in disease is too weak at the moment.

MCPyV and MCC

MCC is a rare and aggressive neuroendocrine tumor of epidermal origin with an increasing incidence of approximately 0.2 - 0.5 per 100,000 depending on age, skin type, (cumulative) sun exposure and immune status (**Figure 6A**) [77, 78]. The association with fair skin and sun exposure suggests a direct effect of UV-radiation in MCC development, but this has not been confirmed experimentally. In 2008, the presence of MCPyV in MCC lesions was shown for the first time [9], a finding that has been confirmed by many research groups worldwide showing that at least 75% of all MCCs have monoclonally integrated MCPyV genome [65].

Several findings indicate direct involvement of MCPyV in Merkel cell carcinogenesis. For instance, in almost all cancer cases MCPyV is found linearized and monoclonally integrated in the host tumor-cell genome, although the site of integration appears random [9, 44, 79]. Furthermore, the MCPyV large T-gene was found to carry 'signature' mutations specific for the carcinomas. Their expression results into truncated large T-antigen that lacks the C-terminal helicase domain, the putative p53-interaction domain and the origin-binding domain [9, 55, 80], consequently rendering the virus inactive for replication. Therefore, the function of large T-antigen in viral progeny production is selectively impaired. Small T-antigen is unaffected by these mutations. Therefore, in an apparent parallel to other virus-associated cancers, e.g. human papillomavirus (HPV)-associated cervical cancer, also in MCPyV-positive MCC there seems to be a strong association with non-productive MCPyV infection. Probably the strongest evidence of pathogenicity (oncogenicity) of MCPyV is provided by studies showing that expression of both small and (truncated) large T-antigens are indispensable in the maintenance of cancer cell phenotype [81, 82].

TSPyV and trichodysplasia spinulosa

Trichodysplasia spinulosa (TS) is a rare skin disease exclusively found in severely immunocompromized hosts, especially the solid organ transplant recipients (**Figure 6B**) [85, 86]. TS is characterized by gradual development of papules and spicules (spines) on the face, sometimes accompanied by alopecia of the eyebrows and lashes [87]. Worldwide, some 30 cases have been described so far [88, 89]. In 1999, Haycox and co-workers proposed for the

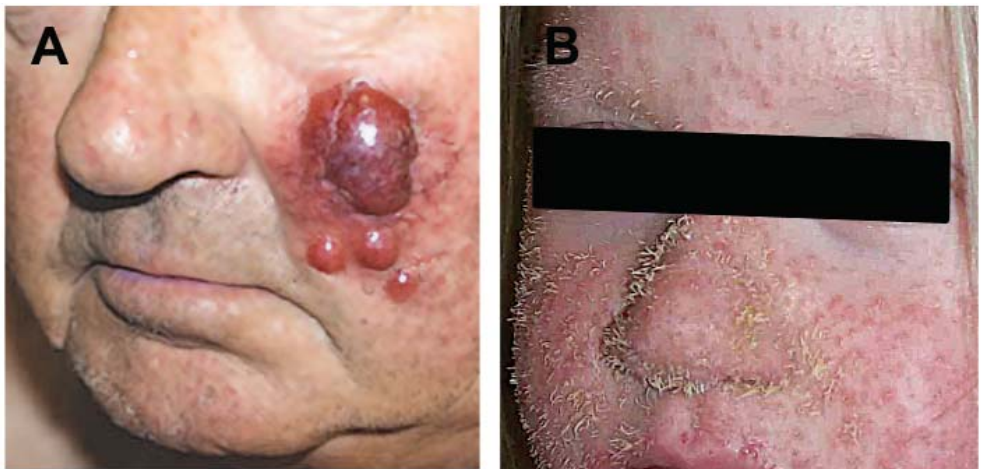

Figure 6. (**A**) Presentation of a 5-cm large Merkel cell carcinoma with concomitant nodal spread and highly vascularized surface on the left cheek of a 72-year-old man [83]. (**B**) Presentation of trichodysplasia spinulosa showing eyebrow alopecia, spiny spicule eruptions and multiple inflammatory papules on the face of a 5-year-old immunocompromized girl [84].

first time that trichodysplasia spinulosa concerns a viral-associated disease and showed the presence of intranuclear clusters of honeycomb-arranged viral particles in the distended and dysmorphic hair follicles [46]. Subsequent reports confirmed the typical histological findings of hyperplastic hair bulbs and the presence of polyomavirus or papillomavirus-like particles [90 - 94]. Attempts to identify the nature of this virus failed until TSPyV was identified with the help of RCA in 2010 [10]. More detail about TSPyV's virological and pathogenic properties, as well as epidemiologic, diagnostic, and therapeutic aspects will be presented in **Chapter 2**.

Prevalence, persistence, reactivation and spread of polyomavirus infection

Seroprevalence

Cross-sectional, serological studies of JCPyV and BKPyV have shown that both infections are highly prevalent in the general population. The seroprevalence of JCPyV is approximately 60% and of BKPyV approximately 90% [47]. Serological studies of the novel human polyomaviruses revealed a considerable variation in the presence of serum antibodies [14, 47, 95 - 101]. The lowest seroprevalence was detected for HPyV9 varying between 15% and 45% [100, 102, 103], the highest for WUPyV, MCPyV, TSPyV and HPyV6 varying between 60% and 95% [14, 47, 97, 98, 104]. In all serological studies, the strongest increase in seroprevalence is observed at a young age, indicating that primary infections generally occur at (early) childhood [47, 96, 98, 99]. So far, the seroprevalences of the novel HPyVs measured with VP1-VLPs and GST–VP1 fusion proteins appear similar, as was shown for instance for TSPyV [97, 98].

Persistence and reactivation

It is generally believed that primary polyomavirus infection is followed by a persistent, asymptomatic (latent) infection with very low levels of viral replication, which could remain hidden in the body lifelong. For JCPyV and BKPyV reactivation from the latent state and manifestation of symptomatic infection is observed only in the case of reduced immunity. For instance, nephropathy can develop in long-term immunosuppressed solid organ transplant recipients caused by BKPyV, and PML in AIDS patients and Natalizumab-treated multiple sclerosis patients caused by JCPyV [52, 105, 106].

For most polyomaviruses, in healthy individuals the detection rate of viral DNA is much lower than their seroprevalence. In urine of healthy blood donors for example, the detection rate of JCPyV and BKPyV DNA was approximately 3- and 10-fold lower than the seroprevalence measured within the same group, respectively [107]. In immunocompromized patients, however, BKPyV can be detected in urine at very high loads indicative of massive reactivation of infection and shedding of the virus [108, 109].

For the novel HPyVs, the association between diminished immunity and reactivation is less clear. Only for TSPyV, with a seroprevalence of approximately 75% [97, 98, 103], clinical consequences are seen exclusively in severely immunocompromised hosts (see also **Chapter 2**) [85, 86]. However, the TSPyV DNA detection rate, on the skin at least, hardly varies among immunocompetents (2%) and immunocompromised hosts (4%) [10, 85]. Also for MCPyV, the DNA detection rates on the skin do not differ much between immunocompetents and immunocompromized, albeit they are generally much higher compared to TSPyV and resemble the seroprevalence of about 50% [110]. In general, it should be kept in mind that both MCC and TS are very rare conditions implying that (host) factors other than immunity may also play a role in controlling these infections. For KIPyV, WUPyV HPyV6, HPyV7, HPyV9, MWPyV, HPyV10, MXPyV, STLPyV, HPyV12 and NJPyV infections, the relation with immunity is not (yet) solved. This is largely explained by weak disease associations or the lack of an established clinical condition that could be attributed to one of these novel viruses and the unawareness of the organ where these viruses could latently persist.

Spread of infection

Earlier studies have shown that JCPyV and BKPyV are found in urine, feces and saliva. The excreted virus numbers are so high that these polyomaviruses even serve as markers of sewage pollution or recreational waters [111]. Which route of virus excretion primarily drives human infection is not precisely known, but the efficiency of infection seems clear from the high seroprevalence, for both the known and novel human polyomaviruses.

Regarding possible routes of excretion and transmission of cutaneous polyomaviruses, a recent study showed the presence of MCPyV DNA on 75% of samples from environmental surfaces (door handles, ticket machines etc.). Prior DNase treatment of the samples and subsequent viral load measurement suggested that about 5% of the detected MCPyV DNA was protected, most probably encapsidated, and therefore potentially infectious [112]. If this provides a solid clue regarding the virus transmission, remains to be seen.

Phylogeny and evolutionary trends

So far, the thirteen known HPyVs comprise the largest host-specific subset of identified polyomaviruses. This probably reflects a bias of the virus discovery efforts that increasingly focus on hunting for human viruses, particularly during the past several years as was detailed above and shown in **Figure 2**. Pairwise divergence between HPyVs varies as much as in the entire polyomavirus family. If measured for the combined conserved regions of VP1, VP2 and LT proteins it ranges from 15% (for BKPyV/JCPyV pair) to 74% (KIPyV/NJPyV), or, if repeated substitutions accounted, from 0.16 (BKPyV/JCPyV) to 2.08 (KIPyV/NJPyV) substitutions per position on average (**Figure 7**). Apart from KIPyV/WUPyV, also the HPyV6/HPyV7 and BKPyV/JCPyV pairs are among the most closely related, which correlates with putative shared tropism for the respiratory tract, skin and the urinary tract infection, respectively.

In **Figure 8**, a tentative polyomavirus phylogenetic tree is illustrated. In addition to the older tree shown in **Figure 1** [2], the novel tree includes several newly identified animal and human viruses that represent new putative polyomavirus species. From this tree, it is

	KIPyV	WUPyV	HPyV6	HPyV7	BKPyV	JCPyV	TSPyV	MCPyV	HPyV9	STLPyV	MWPyV	HPyV12	NJPyV
KIPyV	0	0.23	0.55	0.52	0.57	0.60	0.62	0.61	0.61	0.62	0.61	0.63	0.74
WUPyV	0.27	0	0.54	0.53	0.56	0.57	0.62	0.60	0.62	0.59	0.61	0.61	0.73
HPyV6	0.92	0.91	0	0.23	0.63	0.62	0.64	0.59	0.63	0.61	0.63	0.62	0.71
HPyV7	0.87	0.88	0.26	0	0.61	0.62	0.62	0.60	0.62	0.61	0.61	0.63	0.71
BKPyV	1.04	0.99	1.26	1.19	0	0.15	0.44	0.48	0.46	0.49	0.47	0.48	0.69
JCPyV	1.12	1.02	1.20	1.19	0.16	0	0.45	0.49	0.47	0.49	0.47	0.46	0.69
TSPyV	1.21	1.20	1.29	1.19	0.64	0.66	0	0.39	0.35	0.47	0.42	0.38	0.63
MCPyV	1.17	1.12	1.13	1.12	0.74	0.76	0.54	0	0.42	0.51	0.49	0.41	0.61
HPyV9	1.17	1.19	1.26	1.21	0.71	0.71	0.47	0.60	0	0.47	0.45	0.41	0.68
STLPyV	1.21	1.12	1.18	1.17	0.76	0.76	0.70	0.81	0.69	0	0.33	0.48	0.70
MWPyV	1.16	1.16	1.23	1.16	0.72	0.71	0.60	0.76	0.67	0.42	0	0.45	0.69
HPyV12	1.21	1.16	1.22	1.23	0.72	0.70	0.51	0.58	0.56	0.72	0.67	0	0.67
NJPyV	2.08	2.01	1.99	1.92	1.76	1.74	1.40	1.31	1.62	1.71	1.75	1.60	0

0.00 - 0.49
0.50 - 0.99
1.00 - 2.10

Figure 7. Genetic comparison between thirteen human polyomaviruses is shown. Two measures of genetic distance are shown for the combined polyomavirus protein-identity of conserved regions of VP1, VP2 and LT proteins. The upper-right triangle shows uncorrected dissimilarity percentages (1 corresponds to 100% dissimilarity; 0 means identical sequences); the lower-left triangle shows evolutionary distances that correct for multiple substitutions at the same site (the scale is mean substitutions per sequence position) under the WAG amino acid substitution model and rate heterogeneity among sites [113] (C. Lauber is acknowledged for updating the figure).

clear that the HPyVs do not form a monophyletic cluster. Rather, they are distributed unevenly among four out of five distant lineages. Two lineages that include human polyomaviruses belong to the genus *Orthopolyomavirus* [2], and are called *Orthopolyomavirus-I* and *-II*, respectively. The third lineage belongs to the genus *Wukipolyomavirus*, and the fourth comprising the genus *Malawipolyomavirus* includes MWPyV (as well as HPyV10 and MXPyV that are almost identical to MWPyV) and STLPyV all isolated from human stool.

The *Malawipolyomavirus* and Wukipolyomavirus lineages are the least populated and include exclusively HPyVs. In contrast, the *Orthopolyomavirus-I* and *-II* include 45 putative species from which five and two, respectively, are composed of HPyVs. Because of the observed phyletic distribution of HPyVs among different lineages, it may be envisaged that a number of human polyomaviruses emerged because of cross-species jumps of zoonotic viruses rather than of virus-host coevolution [114, 115]. Recently, the applicability of this virus-host coevolution model to two species of best-sampled polyomaviruses, BKPyV and JCPyV [116 - 118], as well as the entire family [119] was questioned.

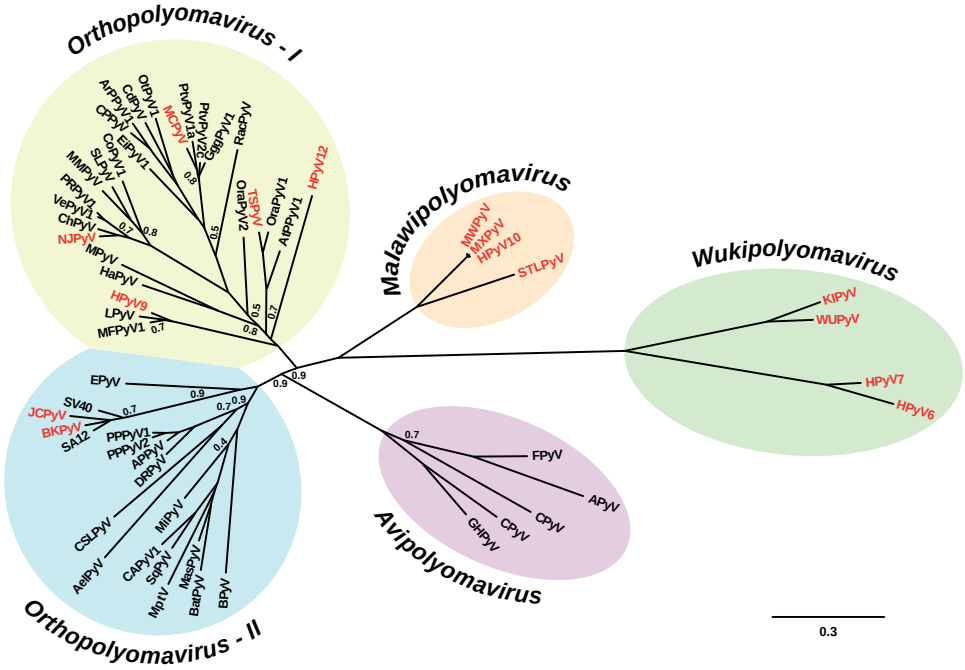

Figure 8. Unrooted phylogenetic tree (up until June 2014) of known (putative) polyomavirus species based on the alignment of concatenated VP1, VP2 and LT amino acid sequences. The obtained branching pattern (topology) of basal nodes in the tree matches that proposed by Johne and colleagues [2], shown in **Figure 1**. HPyVs are shown in red. The bar indicates number of substitutions per site. The numbers at branching events represent probability support values ranging from 0 (no support) to 1 (best support). Only support values lower than 1 are shown. The full polyomavirus names can be found in **Table 1** (C. Lauber is acknowledged for updating the figure).

Resolving the uncertainty about the origins of human polyomaviruses will require extending the virus discovery efforts. For example, continuation of hunting for human viruses and characterization of other hosts should lead to considerable improvement in our understanding of polyomaviruses (host) evolution. Moreover, additional polyomavirus studies regarding their gene expression and overprinting mechanisms will facilitate understanding of polyomavirus-specific patterns of speciation and host adaptation.

Concluding remarks

With the availability of sensitive molecular techniques to detect unknown genome sequences in the background of human genomic DNA, the discoveries of polyomaviruses have increased significantly over the past few years. It is expected that these discoveries will continue in the coming years.

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Outline and Scope of this dissertation

In **Chapter 1**, the focus was on the recent developments in studying the newly identified human polyomaviruses, which reviewed several general aspects of virus identification, pathogenicity, epidemiology and complete *Polyomaviridae* family phylogeny. Still, detailed analyses of these aspects for each virus are desperately needed. This dissertation will address some of these issues in particular for TSPyV.

In **Chapter 2**, the virological and clinical aspects of TS disease are discussed in more detail, including epidemiologic, diagnostic, and therapeutic aspects of TSPyV infection.

To study causality between TSPyV infection and TS disease, in **Chapter 3** the prevalence, load and localization of TSPyV infection in a series of TS patients is analyzed in comparison to healthy controls.

In **Chapter 4**, it is investigated which cellular mechanisms are potentially disrupted by TSPyV, thereby contributing to hyperproliferation of infected hair follicle cells and spicule formation. Furthermore, the putative role of TSPyV Large T-antigen in this process is studied.

In **Chapter 5**, we (re)assessed *in-silico* the genome sequences of all available (recently identified) polyomaviruses known so far. A newly identified ORF encoding MT/ALTO-antigen is characterized in more detail, and its role in polyomavirus evolution and adaptation is addressed.

In **Chapter 6**, whole genome sequences of TSPyV are obtained and analyzed from a series of TS cases described in Chapter 3, for the purpose of gaining insight into the evolution of this polyomavirus. The phenomena regarding polyomavirus evolution described in Chapter 5 are employed on TSPyV evolution that acts as a unique *in-silico* model to study the molecular basis of amino-acid exchange and toggling in polyomavirus host adaptation in general.

Finally, in **Chapter 7**, the findings described in this dissertation will be discussed with regard to TSPyV infection, pathogenesis, evolution and host adaptation, and compared to the existing knowledge about polyomaviruses in a broader context. In addition, some thoughts about future research directions will be shared in this chapter.

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