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Novel polyomavirus in the endangered garden dormouse *Eliomys quercinus*

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Abstract

Background The garden dormouse (*Eliomys quercinus*) has experienced a significant population decline across Europe in recent decades. While habitat loss and climate change are often cited as primary factors, pathogen exposure, either to novel or to previously known, may play a role in such a decline. This study aimed to investigate the presence of polyomaviruses in garden dormice, given that these viruses are highly prevalent and can cause disease, particularly in immunocompromised individuals.

Methods The carcasses of garden dormice ($n=89$) were collected throughout Germany. Kidney samples were tested for the presence of polyomavirus DNA using nested degenerate and specific diagnostic PCRs. Seroprevalence was assessed from chest cavity fluid samples through an enzyme-linked immunosorbent assay using polyomavirus VP1 virus-like particles produced in yeast.

Results A new polyomavirus, related to chimpanzee (*Pan troglodytes*) polyomaviruses 4 and 5 and human Merkel cell polyomavirus, was identified in the garden dormouse. Two 5,380 bp-length complete viral genomes were sequenced from dormice kidney samples (sequences PQ246041 and PQ246042). Genes encoding the putative structural proteins VP1, VP2, and VP3, as well as the Large, Middle, and small T antigens, containing conserved functional domains were identified. Polyomavirus DNA was detected in 2 of 74 dormice (2.7%, 95% confidence interval: 0-6.4%) through PCR, while 12 of 69 animals (17.4%, 95% confidence interval: 8.4-26.3%) tested positive for polyomavirus-specific antibodies.

Conclusions In conclusion, here we describe a novel polyomavirus in the garden dormouse with molecular and serological detection. Pairwise sequence comparison and phylogenetic analysis suggest that this novel virus may represent a novel species within the genus *Alphapolyomavirus*. Future work should examine if this virus is garden dormouse-specific and whether it is associated with disease in dormice.

Keywords Polyomavirus, Seroprevalence, Garden dormouse, Infection, Virus-like particles, Complete genome, Phylogeny

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Introduction

The geographical range of the garden dormouse (*Eliomys quercinus*) in Europe has been decreasing during recent decades. In 2015, these animals covered only 49% of the species' 1978 geographical range and 67% of its 2008 range [1]. The garden dormouse is considered extinct or is present in small, isolated populations in the northern and eastern parts of Europe and its populations are declining in central Europe as well. The southwestern parts of the continent retain larger populations of the species. The species is currently listed as "Near Threatened" by the International Union for Conservation of Nature (IUCN) but a change to the status "Vulnerable" has been proposed because of the ongoing range contraction [1, 2]. In Germany, the species is classified as "Endangered." The reasons for the accelerating declines in large parts of its range remain unknown, and therefore no specific risk factor can be identified [3]. To investigate possible reasons of such decline in the region, a project "In Search of the Garden Dormouse" was launched in Germany in 2018 [4]. Numerous dormouse carcasses were collected by volunteers (citizen scientists) and examined. The variables investigated included predators, pathogens, pollutants, pesticides, and rodenticides, as well as genetic factors [5, 6, other manuscripts in preparation]. However, beyond the known dormice pathogens, such as *Borrelia* spp., which have not been directly linked to the species' decline, garden dormouse may also carry other common and disease-associated pathogens that are yet to be identified in this species.

Polyomaviruses (PyVs) are small double-stranded DNA viruses with wide animal host range, from vertebrates to arthropods [7]. Several PyV-related oncological and other diseases were described in immunocompromised humans, other mammals and birds, e.g., BK polyomavirus associated nephropathy in kidney transplant recipients or lymphomas in newborn Syrian hamsters [8, 9]. However, PyVs are typically detected in healthy hosts without pathological implications [10, 11]. The number of described PyVs increased rapidly in recent years – in year 2024 alone, over 80 PyV-like complete genome sequences recovered from various animal species were deposited to the National Center for Biotechnology Information (NCBI), including several rodent associated sequences. Novel PyVs were reported in Eurasian beaver (*Castor fiber*) and marmots (*Marmota caudata*, *Marmota marmota*), bringing the total number of rodent PyVs with complete genomes available to 26, with 16 currently being confirmed as species by the International Committee on Taxonomy of Viruses (ICTV) [12–29]. Considering a number of 2000 species comprising the order Rodentia, it is, however, still the worst represented mammalian order in terms of PyV detection. More than a dozen human-specific PyVs infect people, with varying prevalences [30, 31]. This suggests that more comprehensive surveys of rodents are needed to evaluate the diversity of PyVs.

Polyomavirus seroprevalence has been thoroughly investigated in human populations of different ages and geographic locations, with 17–100% seropositivity for the most established human PyVs 1–10 (HPyVs 1–10). The very low seroprevalence (<5%) of proposed HPyVs 12 and 14 related to the realization that these viruses either represented PCR contaminants or rare PyV spillover infection events, originating from wild common shrews (*Sorex araneus*) and pet cats (*Felis catus*), respectively [32–36]. Seroprevalence has only been reported for three rodent-associated PyVs, and it ranged from 7 to 10% in wild voles (common vole *Microtus arvalis*, CVPyV, and bank vole, *Clethrionomys glareolus*, BVPyV), to 32% in laboratory rats (Norway rat *Rattus norvegicus*, RatPyV2) [24, 37]. PyV DNA detection in blood was identified to be very low (0-3.8%) in healthy human blood donors, but the prevalence of cutaneous HPyV DNA on the skin ranged from 0.9% (HPyV8, Trichodysplasia spinulosa-associated polyomavirus, and HPyV9) to 67.9% (HPyV5, Merkel cell polyomavirus) [10, 38]. The prevalence of PyV DNA (0.5–67%) is described for less than half of the known rodent associated PyVs and in most cases a single geographic region coupled with a relatively small number of animals was evaluated [16–18, 20, 22–24, 37]. While the knowledge on human PyV infection has been growing and PyV role in disease has been increasingly explored, investigation of PyV infection in wild animals is superficial.

Polyomaviruses are known for their ability to cause oncogenic transformation, mainly through the actions of their tumor (T) antigens: Large T (LTAg), small T (sTAg), and Middle T (MTAg) [39, 40]. LTAg drives viral replication by inactivating the retinoblastoma protein (pRb) and initiating viral DNA replication, while sTAg inhibits phosphatase 2 A (PP2A) to stop cell cycle progression. MTAg, located in the cell membrane, interacts with PP2A and kinases to regulate signaling pathways involved in cell growth and immune response [41–44]. T antigens possess highly conserved functional domains that enable precise modulation of the host cell cycle and proteome, which typically results in the mild, latent infections seen for polyomaviruses [45]. However, when the virus switches to a new host species, increased pathogenicity can occur, as observed with Budgerigar fledgling disease virus (*Gammapolyomavirus avis*) [46]. Accurate, multimethod virus detection, alongside confirming its absence in sympatric species, is essential for assessing host specificity and disease potential.

In this study, we report the complete genome of a novel PyV detected in garden dormouse. We developed virus-specific molecular and serological assays and provide initial (sero)prevalence data for this virus in wild garden

dormice collected at different sites in Germany. This is the first report of a PyV infection in garden dormouse which emphasizes the need for further surveillance of these pathogens in dormice and sympatric species.

Materials and methods

Sample collection

Garden dormice found dead were collected from almost the complete known range of the species in Germany as part of a German-wide project called "In Search of the Garden Dormouse" [47]. Coordinated by employees of the non-governmental organization "Friends of the Earth Germany", mainly citizen scientist volunteers sampled garden dormouse carcasses found dead in gardens, on public property, in traps, in nest boxes or brought home by cats. The eight carcasses from Saxony came from animals held in captivity. All carcasses were immediately frozen and transferred to the Clinic for Birds, Reptiles, Amphibians and Fish (Gießen, Germany). The carcasses were collected in 2012 ($n = 1$) and between 2016 and 2020 ($n = 88$) (Table S1). Chest cavity fluid (CCF), if present, was collected with a syringe from the animal's chest cavity after the freeze-thaw of the carcass. Kidney samples were dissected and stored at -20 °C.

DNA extraction, enrichment and PyV identification

Frozen kidney samples (approximately 20 mg) were lysed in 200 µl of lysis buffer (50 mM Tris-HCl, 0.15% SDS, 3 mM $CaCl_2$, 150 mM NaCl) with 5 µl of proteinase K (20 mg/ml, Thermo Scientific) at 56 °C for 4 h. DNA was extracted from the lysate using the GeneJET Viral DNA and RNA Purification Kit (Thermo Scientific). The extracted DNA was divided into two parts: one half was directly used for PCR; the other half underwent precipitation at -20 °C using 7.5 M NH_4OAc and 96% ethanol. The precipitated DNA was then enriched for circular molecules using rolling circle amplification (RCA) with EquiPhi29 polymerase, exoresistant random primers, and pyrophosphatase (Thermo Scientific). Broad-range nested PCR targeting the conserved part of the PyV LTAg encoding region was conducted as previously described [48]. The resulting DNA fragments of 220 base pairs (bp) in length were inserted into the pJET1.2 cloning vector (Thermo Scientific) and dideoxy-chain termination Sanger sequencing was performed by GENEWIZ (Azenta Life Sciences, Leipzig, Germany).

Complete genome determination of the novel polyomavirus

The full genome of the identified PyV was amplified using Phusion Green High-Fidelity DNA Polymerase (Thermo Scientific) with back-to-back EquePyV-LT-R/D primers (Table S2), inserted into the pJET1.2 cloning vector (Thermo Scientific) and sequenced by GENEWIZ (Azenta Life Sciences, Leipzig, Germany).

Diagnostic PCR for detection of the novel polyomavirus DNA

To detect the dormouse PyV DNA, diagnostic PCRs were conducted on both RCA-enriched and unenriched samples using DreamTaq Green DNA Polymerase (Thermo Scientific) with specific diagnostic primers targeting LTAg encoding sequence (Table S2).

Virus-like particle production and imaging

Production of virus-like particles (VLPs) of the novel PyV followed established protocols for other PyVs [24, 49]. The VP1 gene from sample 155 was amplified with Phusion polymerase (Thermo Scientific) using VP1-specific primers (Table S2) and cloned into the yeast expression vector pFX7 [50]. The plasmid was transformed into *Saccharomyces cerevisiae* strain AH22-c214 (*a*, *leu2-3,112*, *his4-519*), protein synthesis was induced with 3% galactose. VP1 VLPs were purified via ultracentrifugation through sucrose and cesium chloride gradients after yeast biomass was mechanically lysed with 0.5 mm diameter glass beads (Sigma-Aldrich) in Disruption Buffer (10 mM Tris-HCl (pH=7.2), 450 mM NaCl, 0.01% Triton-X-100 (v/v), 1 mM $CaCl_2$, 0.25 M L-Arg, protease inhibitor phenylmethylsulfonyl fluoride 2 mM, protease inhibitor cocktail tablet (Fisher Scientific). Fractions containing VP1 were identified via SDS-PAGE, further purified, and dissolved in phosphate-buffered saline (Roth). Samples were dialyzed to remove small proteins and salts and stored at -20 °C in 50% glycerol (Fisher Scientific). Glycerol stocks were diluted and stained with uranyl-acetate for transmission electron microscopy with Morgagni 268 (FEI Inc.).

Antibody detection and seroprevalence determination

To assess the seroprevalence of the novel PyV in dormouse CCF samples, an indirect ELISA was performed using purified VP1-derived VLPs as the antigen. F-shaped polystyrene plates (Cat# 10-121-0000, nerbe plus) were coated with 100 µl of a 2 µg/mL VP1 VLP solution in washing buffer (10 mM Tris, 0.3 M NaCl, 0.1% Tween-20, pH 7.2) per well and incubated overnight at 37 °C. After washing three times, wells were blocked with 3% bovine serum albumin (Gibco) in washing buffer for 1 hour. Dormouse CCF samples were diluted (1:50, 1:100, 1:200), with anti-*Alphapolyomavirus muris*-VP1 monoclonal antibodies (1:800) as a positive control and hamster (*Cricetus cricetus*) serum as a negative control. After a 1-hour incubation at 37 °C, plates were washed, and goat anti-mouse IgG-horse radish peroxidase conjugate (1:10,000, Cat# 172–1011, Bio-Rad) was added, followed by a second 1-hour incubation. After washing, 3,3',5,5'

tetramethylbenzidine (Life Technologies) substrate was added, and the reaction was stopped after 15 min with 10% sulfuric acid (Sigma). Absorbance was measured at 450 nm using an Infinite M200 plate reader. The experiment was conducted in two replicates, the cutoff value for each plate was determined based on the negative control average plus three times standard deviation (see Table S7 for details).

Bioinformatics and statistical analyses

The results of Sanger sequencing were processed, and novel viral genomes were annotated using Benchling software [51]. Early region splicing donor and acceptor sites were predicted with ASSP tool [52] (details in Table S3). VP3 sequence was deduced by presence of established MALXXΦ (Φ=W, F, Y) motif [53]. Presence of conserved protein motifs was evaluated with ScanProsite tool [54], nuclear localization signals (NLS) determined with NLS mapper [55], potential SH2 and SH3 binding sites with ScanProtein [56], and transmembrane sequence motifs with DeepTMHMM [57].

Up-to-date PyV complete genome sequences of 3800 to 8000 bp in length were downloaded from NCBI (n=2727, as of 2024-07-23), and clustered with CD-HIT 4.8.1 [58] to 90% identity, keeping a single representative for each high identity group. ICTV recognized PyV species' representatives and a single representative with complete genome sequence available were selected for further analysis. Then sequences were manually inspected, and gene annotations and splicing were adjusted respective to sequence alignments and published data [17]. The final dataset consisted of 282 PyV sequences (Table S4). Extracted LTAg and VP1 amino acid (aa) sequences were aligned with MAFFT v7.526 [59], phylogenies were inferred with RAxML-NG v. 1.2.0 [60] (20 starting trees, model LG+G+I+F, autoMRE with 0.03 cutoff and Transfer Bootstrap Expectation support metric [61]) and visualized using iTOL [62].

To assess the overall PyV occurrence in dormice, Sequence Read Archive (SRA) data (concatenated reads) for the rodent family Gliridae (n=106, 2024-08-09, Table S5) were scanned using Diamond 2.1.9 [63] blastx option against the created LTAg and VP1 protein baits of 282 PyVs. Matched reads were analyzed with NCBI blast. Dormouse PyV genome sequence was also used for broad database screens with recently developed tools Pebblescout [64] and MetaGraph [65].

Confidence intervals (95%) for PyV (sero)prevalence were determined as:

$$\mathrm{SE} = \sqrt{\frac{p(1-p)}{n}},$$
$$\text{Lower Bound} = p - z \times \mathrm{SE},$$
$$\text{Upper Bound} = p + z \times \mathrm{SE}.$$

Where:

- SE – Standard Error;
- p – proportion of positive cases;
- n – total number of cases;
- z – Z-score for a 95% confidence interval (Z = 1.96).

For situations where the prevalence is 0, the Clopper-Pearson approximation method was used to determine the upper bound:

$$\text{Upper Bound} = 1 - (1-\alpha)^{\frac{1}{n}}$$

Where:

- α – significance level for a 95% confidence interval, α = 0.05;
- n – total sample size.

For details see Table S7.

Results

Identification of a novel polyomavirus in the garden dormouse and its genome characterization

Broad-range nested PCR on DNA extracted from kidney samples of dead garden dormice and RCA-enriched in circular DNA molecules resulted in products of the expected size (approximately 220 bp) in two of 74 samples (samples 155 and 224). These amplification products were sequenced and aligned with NCBI nucleotide blast tool, which showed PyV DNA sequences as closest matches. Use of back-to-back primers resulted in the whole PyV genome amplification from both samples.

Sequenced genomes from garden dormouse samples 155 (PQ246041, EquePyV-155) and 224 (PQ246042, EquePyV-224) are both 5380 bp in length and have open reading frames (ORFs) for putative structural VP1, VP2, and VP3 in the late coding region, as well as LTAg, sTAg, and MTAg in the early coding region (Fig. 1, putative encoded genes are described in more detail in Table S6). All T antigen coding sequences start at the same ATG, however, predicted splicing of sTAg mRNA results in a switch to the +2 frame and that of LTAg mRNA to the +3 frame. In contrast to other PyVs, which encode MTAg on a spliced mRNA, the inferred EquePyV MTAg is a single contiguous ORF. Early and late regions are separated by a regulatory region of 678 bp. The two PyV genomes differ by seven nucleotides, of which five are synonymous changes. These include non-silent changes in the MTAg

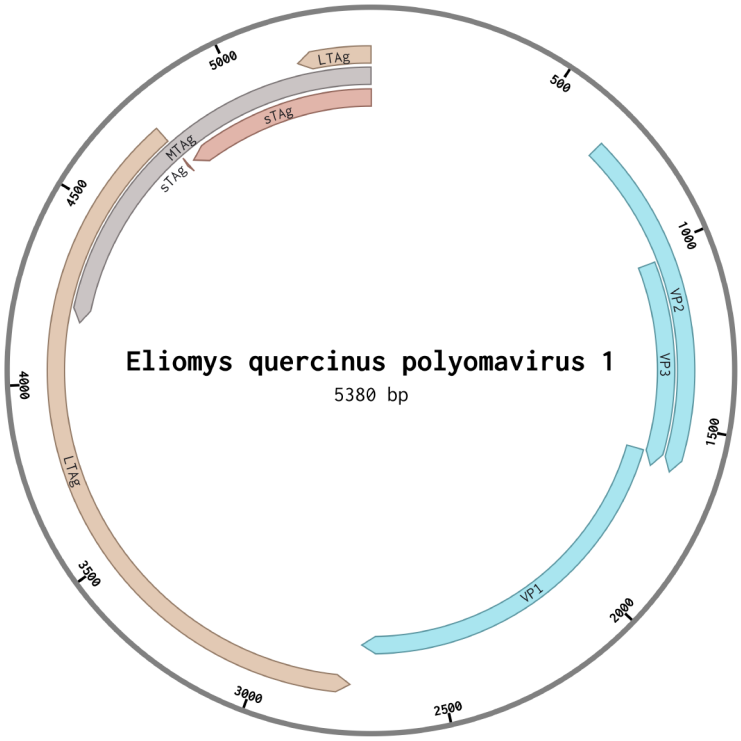


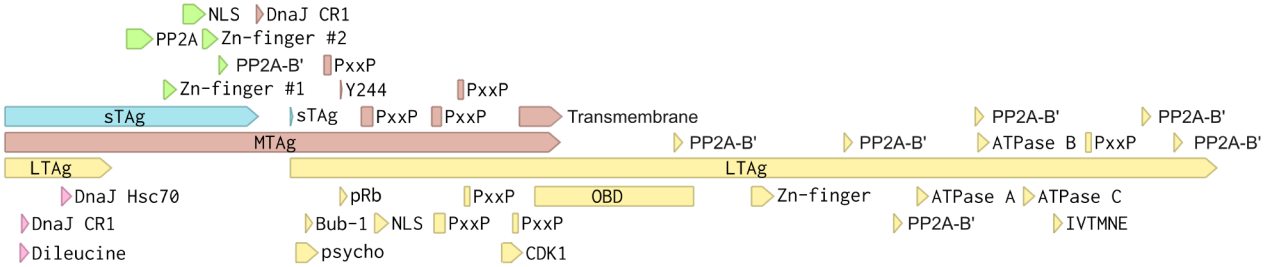
Fig. 1 Genome organization of the novel garden dormouse polyomavirus. Polyomavirus genome sequenced from wild garden dormouse (*Eliomys quercinus*). Putative late (structural viral proteins, VP1-3) and early (Tumor antigens) genes are indicated in blue (forward) or shades of brown (reverse)

(P238L, V345K) sequence and a single non-silent change in LTAg (S215N). These positions do not overlap with highly conserved regions of PyV T antigens (Fig. 2).

Predicted functional motifs of dormouse PyV T antigens

LTAg, MTAg, and sTAg of the novel PyV were predicted to have the majority of previously described functional motifs for PyV T antigens, responsible for the hijacking of cell replication machinery and cell cycle (Fig. 2) [40, 45, 66]. Highly conserved Rb tumor suppressor protein family binding DnaJ motifs conserved region 1 (CR1) and heat shock cognate 70 (Hsc70) are in the N-terminal part, which is common for all three T antigens. Interestingly, psycho (modulation of Rb binding, name derived from PSY and C residues in the first consensus sequence identified [67]), Bub-1 (mitotic checkpoint serine/threonine-protein kinase binding), ATPase A and conserved ATPase region IVTMNE domains present in dormouse PyV LTAg differ from the published consensus sequences (Fig. 2). Six PP2A B′-family (PP2A-B') binding motifs were identified in the C-terminal part of the LTAg protein, and a cyclin-dependent kinase 1 (CDK1) binding motif was identified in the middle part of the protein. While it was shown that LTAg phosphorylation is important for simian virus 40 (SV40, *Betapolyomavirus macacae*) genome replication and cell transformation, phosphorylation of PyV T antigens remains understudied [68]. It was demonstrated that PP2A-mediated dephosphorylation of LTAg enhances the assembly of LTAg double-hexamer to the SV40 origin of replication and facilitates the unwinding of DNA [69]. On the other hand, the PP2A-B' binding motifs on LTAg might be important for competitively inhibiting substrate access to PP2A, enabling the viral protein to manipulate the host cell's dephosphorylation processes and PP2A modulated cell cycle progression [70]. Four Src Homology 3 (SH3) domain binding motifs were identified in the central part of the LTAg protein (Fig. 2). SH3 domains, which interact with proline-rich sequences, are found in many proteins involved in cell signaling, involving processes such as cell cycle, differentiation and growth [71].

Putative sTAg and MTAg of this virus share PP2A-B', zinc finger (Zn-finger) and NLS motifs. PP2A binding sequence in EquePyV MTAg and sTAg differs from CXXWPXC motif described for SV40 and BK PyVs [66] – it has 14 instead of 2 aa residues between cysteine in the first position and further tryptophan (CakdtknfhtlfiraWPaC). In addition, MTAg of this virus has four SH3 binding domains, one SH2 binding domain and a transmembrane domain in the C-terminal part. These domains are a distinct feature of MTAgs of different alphapolyomaviruses and are important for MTAg membrane binding and phosphorylation followed by cell signaling activation [43].



Viral Protein	Motif name	Published consensus	Sequence	Amino acid position
Large T antigen	Dileucine	[DE]XXXL[LI]	EllqLL	12-17
	DnaJ CR1	LXXLL	LlqLL	13-17
	DnaJ Hsc70	HPDKGG[DN]	HPDKGGD	42-48
	psycho	PTYGTX(9)F	PIYGTX(10)F	82-97
	Bub-1	W[DE]XWW	FRqWW	89-93
	pRb	LXCXE	LrCdE	114-118
	NLS	PK/R-rich	QRPKRRRDTQ	139-148
	SH3 Binding	PxxP, proline rich	PtvPPdPP, PseP, PpkP, PtkP	182-189, 204-207, 239-242, 654-657
	CDK1 binding	[ST]Px[KR]x	TPpKp	238-242
	T antigen origin binding (OBD)	-	-	255-369
	PP2A-B' binding	[LMFI]xx[ILV]xE	FtlLqE, LerLkE, LqlLtE, FavVfE, MeqLrE, FhqLrE	356-361, 479-484, 515-520, 574-579, 695-700, 718-723
	Zn-finger	CX(2)CX(6,9)HX(3)H	CX(2)CX(7)HX(3)H	412-427
	ATPase A	GPX(3)GKT	GAvntGKT	532-539
	ATPase B	VVFEDVKG	VVFEDVKG	576-583
	ATPase C	GX(3)VNLE	GavkVNLE	609-616
	IVTMNE	[ILV]VTMN[ED]	IVTSNE	631-636
Middle T antigen	Dileucine	[DE]XXXL[LI]	EllqLL	12-17
	DnaJ CR1	LXXLL	LlqLL, LgkLL	13-17, 183-187
	DnaJ Hsc70	HPDKGG[DN]	HPDKGGD	42-48
	PP2A	CXXWPXC	CakdtknfhtlfiraWPaC	89-107
	Zn-finger domain #1	CXCX(2)CXL[KR]	CwCvvCmLR	116-124
	Zn-finger domain #2	CXCX(2)CX(3)WF	CfChkCflWF	144-154
	NLS	PK/R-rich	IKKEKKRRCLVWGECF	130-145
	PP2A-B' binding	[LMFI]xx[ILV]xE	LpeLeE	156-161
	SH3 binding	PxxP, proline rich	PenP, PPPPPPtP, PtPPfrP, PrtP	232-235, 259-266, 310-316, 329-332
	SH2 binding	Phosphorylated tyrosine	vlqeegdYvamrpli	Y244
	Transmembrane	Transmembrane helix	VILTLLIFQSVCLIILVTLYIV	377-398
small T antigen	Dileucine	[DE]XXXL[LI]	EllqLL	12-17
	DnaJ CR1	LXXLL	LlqLL, LgkLL	13-17
	DnaJ Hsc70	HPDKGG[DN]	HPDKGGD	42-48
	PP2A	CXXWPXC	CakdtknfhtlfiraWPaC	89-107
	Zn-finger #1	CXCX(2)CXL[KR]	CwCvvCmLR	116-124
	Zn-finger #2	CXCX(2)CX(3)WF	CfChkCflWF	144-154
	NLS	PK/R-rich	IKKEKKRRCLVWGECF	130-145
	PP2A-B' binding	[LMFI]xx[ILV]xE	LpeLeE	156-161

Common for LTAg, sTAg and MTAg
Unique for LTAg
Common for sTAg and MTAg
Unique for MTAg

Fig. 2 Predicted functional motifs encoded by the novel dormouse polyomavirus T antigens. Motifs were identified respective to [66, 70, 71, 77, 85]. Nuclear localization signals (NLS) were identified with https://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi tool, potential SH2 and SH3 binding sites with https://scansite4.mit.edu/#scanProtein, and transmembrane sequence with https://dtu.biolib.com/DeepTMHMM. Mismatches to the known consensus are marked in red

Phylogenetic analysis of the candidate garden dormouse polyomavirus

Novel candidate garden dormouse PyV clusters with PyVs of *Alphapolyomavirus* genus when inferring phylogeny by either LTAg or VP1 aa sequences (Figs. 3 and 4), dormouse PyVs highlighted in green). As shown in Fig. 3, maximum likelihood-based phylogenetic analysis of the LTAg aa sequences placed EquePyVs in a monophyletic group with three primate PyVs (clade consisting EquePyVs sequences marked in red): chimpanzee (*Pan troglodytes*) PyVs 4 and 5 (*Alphapolyomavirus quartipanos*, JX159980 and *Alphapolyomavirus quintipanos*, JX159981) and Sumatran orangutan (*Pongo abelii*) polyomavirus 1 (*Alphapolyomavirus ponabelii*, FN356901). The recently identified Eurasian beaver polyomavirus (OR735477) Identification of a novel polyomavirus in the garden dormouse and its genome characterization is placed in a closest sister clade. Analysis conducted on VP1 aa sequences showed all three primate PyVs in the same clade as EquePyV as well, however, a closest VP1 sister clade contains not only beaver PyV, but also 27 other PyV species from carnivores, bats, even-toed ungulates, primates and human (HPyV5) (Fig. 4). Pairwise LTAg nucleotide sequence alignment revealed that EquePyV LTAg shares 66–67% identity with chimpanzee PyVs 4 and 5, and 64% identity with beaver PyV.

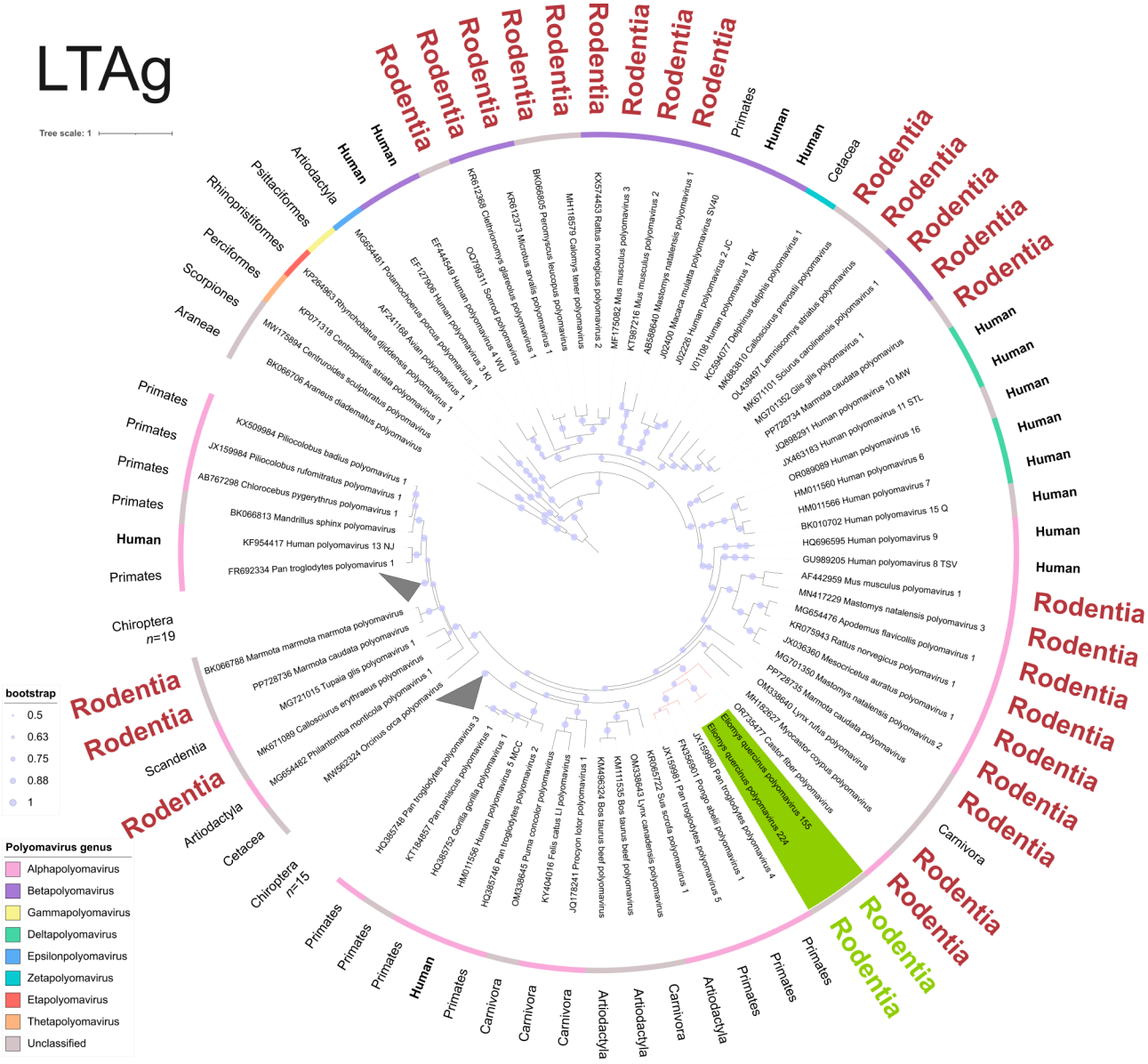


Fig. 3 Polyomavirus phylogeny inferred from LTAg amino acid sequences. LTAg protein sequences of polyomaviruses available in databases (n = 282) and two novel garden dormouse (*Eliomys quercinus*) polyomavirus genomes (labeled in light green) were used for maximum likelihood phylogeny inference. Bootstrapping converged after 300 replicates and transfer bootstrap expectation support metrics with values 0.5-1 are depicted as light blue circles. Viruses are labelled according to the host they are associated with. Virus host order is given in an outside circle label, rodent (order Rodentia) and human associated polyomaviruses are emphasized. Clade with novel dormouse polyomavirus and closest neighbors is marked in red. The tree was pruned to mainly include rodent, human and closest to dormouse polyomavirus PyVs. Protein sequences and complete trees are available in Table S4, and Figures S1 and S2

Dormouse PyV is relatively distant from other rodent alphapolyomaviruses and while there is another PyV previously identified in the edible dormouse (*Glis glis*), it is a distant member of the genus *Betapolyomavirus.*

Prevalence of garden dormouse polyomavirus

Diagnostic PCR with dormouse PyV specific primers, targeting LTAg coding region, was conducted on DNA extracted from kidney samples to determine virus prevalence in dormice. Viral DNA was identified in the same two samples that were identified by nested viral DNA screening before, but not in any other dormouse sample. One dormouse (sample 155) was found dead in Rhineland-Palatinate in 2017. During dissection, a whitish substance was observed in the abdominal cavity of this animal, and its liver exhibited a pale pink coloration. No location metadata is available for the other PCR-positive sample (sample 224), and a centrilobular pattern

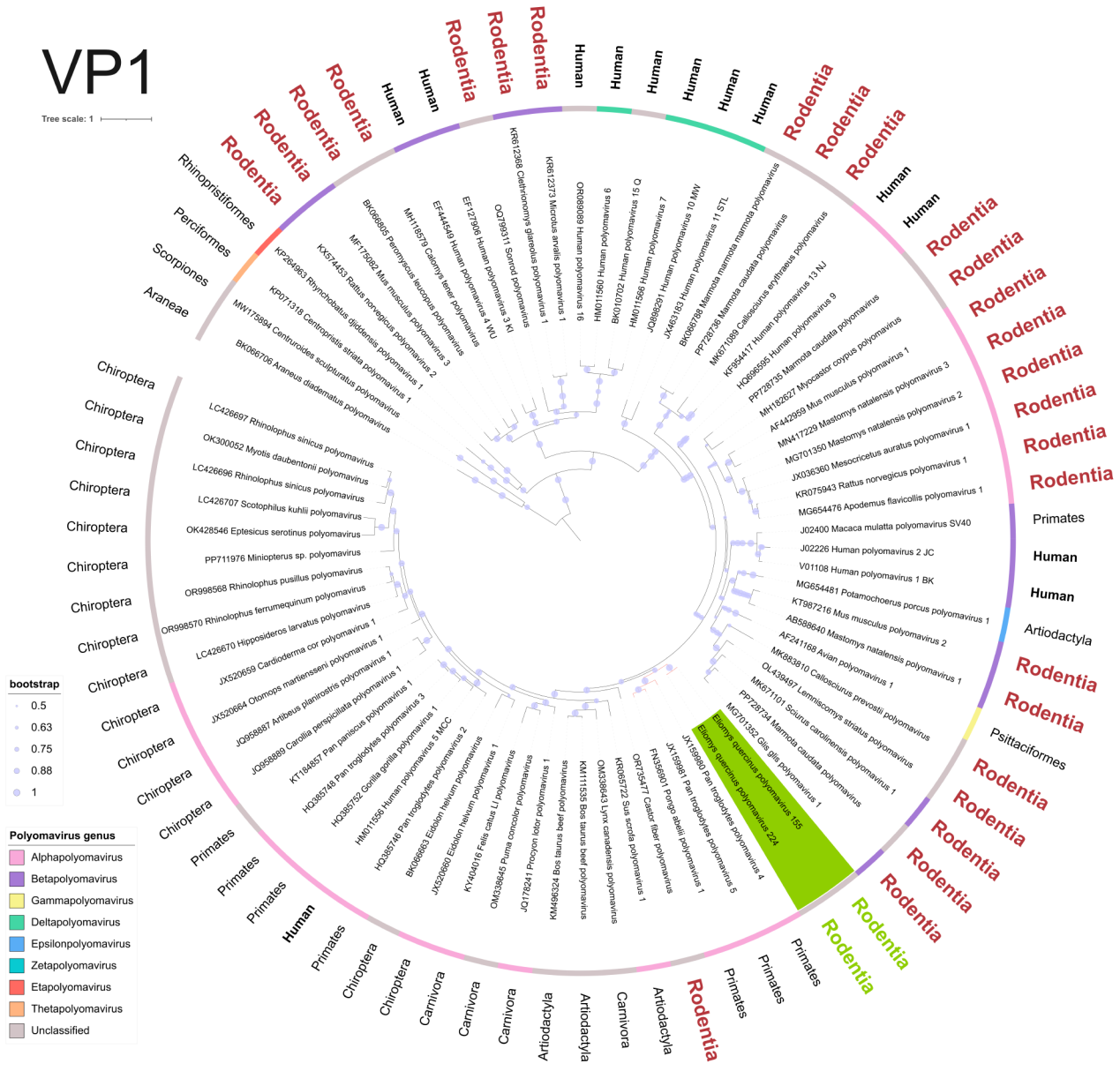


Fig. 4 Polyomavirus phylogeny inferred from VP1 amino acid sequences. VP1 protein sequences of polyomaviruses available in databases (n = 282) and two novel garden dormouse (*Eliomys quercinus*) polyomavirus genomes (labeled in light green) were used for maximum likelihood phylogeny inference. Bootstrapping converged after 560 replicates and transfer bootstrap expectation support metrics with values 0.5-1 are depicted as light blue circles. More details are provided in the legend of Figure 3

was observed in this dormouse's liver during dissection. Overall, viral DNA prevalence was very low in dormice tested – 2.7% (95% confidence interval, CI: 0-6.4%; Table 1).

To determine the seroprevalence of the novel PyV, we synthesized VLPs by expression of EquePyV VP1 in yeast. Obtained particles were non-homogenous, their size ranged from 20 to 50 nm, with majority of the observed particles being around 20 nm in diameter (Fig. 5). Out of 69 tested animal chest cavity fluid (CCF) samples, 12 (17.4%, 95% CI: 8.4-26.3%, Table 1) were seropositive for this PyV. Seropositive animals were identified in all federal states of Germany investigated, with prevalence ranging from 11.1 to 24.1% (95% CI: 0-39.7%). Both animals that tested positive for PyV DNA did not show seropositivity against VP1 VLPs (Table S7).

The screening of over a hundred dormouse SRA datasets, as well as database searches with Pebblescout and MetaGraph, did not result in identification of any additional EquePyV-like sequences (details in Table S5).

Table 1 Polyomavirus prevalence in garden dormice collected in Germany

	PyV DNA prevalence			
Federal state	**neg.**	**pos.**	**Total**	**% positive (95% CI)**
Hesse	17	0	17	0% (0-0.3%)
Rhineland-Palatinate	45	1	46	2.2% (0-6.4%)
Saxony	8	0	8	0% (0-0.6%)
N/A	2	1	3	3.3% (0-8.7%)
Total	**72**	**2**	**74**	**2.7% (0-6.4%)**
	PyV seroprevalence			
Federal state	**neg.**	**pos.**	**Total**	**% seropositive (95% CI)**
Hesse	13	2	15	13.3% (0-30.5%)
Rhineland-Palatinate	21	7	29	24.1% (8.6-39.7%)
Saxony	7	1	8	12.5% (0-35.4%)
N/A	16	2	18	11.1% (0-25.6%)
Total	**57**	**12**	**69**	**17.4% (8.4-26.3%)**

N/A, information missing; neg., negative; pos., positive; CI, confidence interval

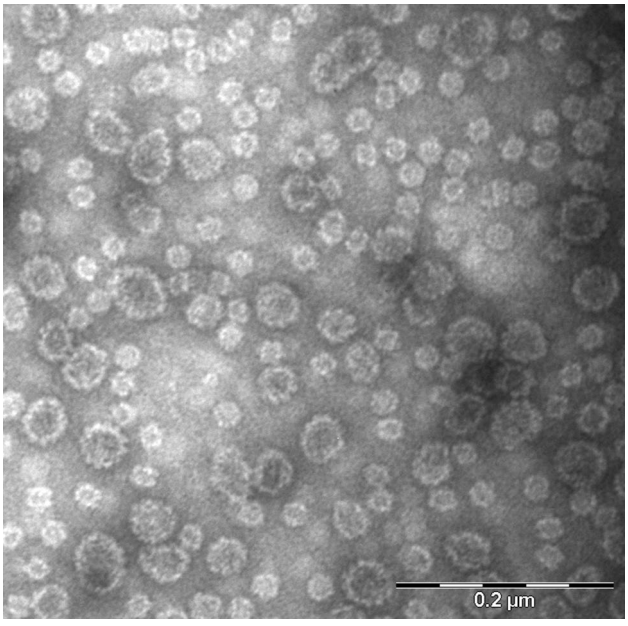


Fig. 5 Electron microscopy picture of yeast-produced dormouse polyomavirus VP1 VLPs. Scale 200 nm. Size range of VP1 virus-like particles (VLPs) is 20-50 nm in diameter

Discussion

In this study, we identified a novel PyV in garden dormouse (Eliomys quercinus polyomavirus, EquePyV). We sequenced the complete genomes of EquePyV from two individual dormice in Germany, finding minimal divergence (seven nucleotides) between the sequences, which is consistent with the highly conservative nature of PyV genomes [72]. Phylogenetic analysis determined that EquePyV forms a clade with three primate PyVs; however, the substantial genetic distance among genomes (~40%) suggests a long evolutionary divergence. Given the slow evolutionary rate of PyVs (approximately 0.5% per million years) [72], this genetic distance implies a host divergence approximately 80 million years ago (MYA), aligning with estimates from the TimeTree database, which places garden dormouse and chimpanzee divergence between 81.3 and 91.0 MYA [73]. This finding supports the hypothesis of virus-host co-evolution, while also highlighting the scarcity of known rodent PyVs related to EquePyV.

The novel virus exhibits genome structure typical for members of *Alphapolyomavirus* genus since it encodes putative structural VP1, VP2, and VP3 and regulatory LTAg, sTAg, and MTAg proteins [43]. The latter proteins were predicted to have the functional domains described for human and animal PyVs, important for PyV replication, hijacking of cell cycle, oncogenesis and persistence [66]. While some T antigen motifs, such as DnaJ, pRb1, and Zn-finger, are highly conserved, others show more variation. For example, the psycho (P**I**YGTX(**10**)F) and Bub-1 (**FR**qWW) domains in EquePyV LTAg exhibit variability in positions previously identified as variable: HPyV5, the human PyV closest to EquePyV, has the sequences P**I**YGTX(**11**)F and **FK**eWW instead of the consensus sequences PTYGTX(9)F and W[DE]XWW [66, 74]. It is unknown whether HPyV5 LTAg can interact with Bub-1, but since the psycho domain is not obligatory for pRb binding, HPyV5 LTAg is still able to bind pRb [74]. The psycho domain is believed to play a role in modulating this binding, although its exact mechanism remains unexplored [74]. This motif, first discovered in the Pur-alpha (purine-rich element binding protein A) protein – which is involved in DNA replication, transcription, translation, and DNA damage repair – may have another crucial function in the PyV life cycle that has yet to be described [75].

The best-studied examples of a PyV MTAg are the main oncogenes of mouse polyomavirus (MPyV1, species *Alphapolyomavirus muris*) and Syrian hamster polyomavirus (*Alphapolyomavirus mauratus*, HaPyV) [76]. MPyV1 MTAg is anchored in the cellular membranes through its hydrophobic region in the C-terminus and possesses binding sites that allow interaction with PP2A and other signal proteins, such as Src homology 2 domain containing protein A (ShcA), phosphatidylinositol 3-kinase (PI3K), and phospholipase C gamma 1 (PLCγ1), and subsequent cell transformation [44]. A recent study of the HPyV5 MTAg-related protein called ALTO (alternative T open reading frame) found that it activates NFκB inflammatory pathway and decreases viral replication and early protein expression, thus functioning as a tumor suppressor – thus serving an opposite function compared to MTAg of MPyV1 and HaPyV [77, 78]. Salisbury and colleagues suggest that ALTO protein, which is expressed from a different frame overlapping with the second exon of LTAg coding sequence, can be differentiated from MTAg, which is usually produced by splicing, by the

presence of N-terminal activating regions (NTARs 1 PXQXXZ and 2 PXQX[ET]) and binding sites for PLCγ1 (YL[DE][IV]) [78]. EquePyV MTAg does not exhibit those sequence motifs and contains SH2 (PI3K p85) binding motif present in MPyV1 MTAg, thus suggesting that it may resemble MTAg more closely than ALTO in function. It remains to be investigated whether this protein has the capacity to transform host cells, as observed with the MTAgs of MPyV1 and HaPyV. Although pathological changes were observed in the livers of the two PyV DNA-positive dormice, no causal link between these pathologies and PyV infection, nor any association with cancerous growth, can be drawn from the current data and warrants further investigation.

Production of PyV VLPs in yeast has been performed in multiple studies for human and animal PyVs and such particles have been used for virus seroprevalence determination [24, 49, 50]. HaPyV has been shown to stably form homogenous particles of around 50 nm in diameter not only from autologous VP1, but also when producing chimeric VP1 (VP1 with foreign epitope insertion in up to 3 positions in the protein), mosaic (co-expression of native and fused with foreign protein VP1) and pseudotype (co-expression of VP1 and VP2 fused with foreign protein) VLPs [79]. However, VP1 of some PyVs, e.g. HPyVs 3, 4, 6, 7 and 9, tend to form heterogeneous VLPs of smaller size in yeast even from native VP1 [49]. This phenomenon was also observed when a longer version of common shrew PyV 1 (*Alphapolyomavirus saraneus*, formerly known as HPyV12) VP1 was expressed, and disappeared when the sequence following the second start codon was used for expression [49], however, EquePyV VP1 coding sequence has only one canonical AUG start codon. It is so far unknown why the abilities of PyVs to assemble into homogenous VP1 VLPs differ, nonetheless, both small and large yeast-derived VLPs induce humoral immune response in mice (with an implication that particles with variable sizes can be strong immunogens) and work well as an antigen for serological assays [80].

So far, PyV seroprevalence has only been determined for a handful of animals, so it is difficult to discuss overall prevalence of these viruses in the wild. Furthermore, several of the rodent PyVs were primarily reported in laboratory animals, some are yet to be reported in wild populations e.g., RatPyV2 had a seroprevalence of 32% in rats from American research institutions but was not detected in wild rats [17, 37]. HaPyV, while occasionally reported in pet hamsters, often in association with pathologies [81], is still the only hamster PyV reported to date and there is virtually no information on its spread in the wild. The observed seroprevalence of dormouse PyV across Germany was 11.1–24.1% (95% CI: 0-39.7%) in different populations. This result is consistent with previous knowledge on PyV seroprevalence in other wild rodents. As shown by Nainys and colleagues, 10% of bank voles and 7% of common voles were seropositive for their corresponding BVPyV and CVPyV [24]. Nonetheless, there are some limitations in determining PyV seroprevalence in our study. These include the use of CCF from frozen, deceased animals instead of fresh serum, along with the anti-mouse secondary antibodies in the immunological assay, which might have contributed to reduced signal strength. Additionally, since the study relied on animal carcasses that may not represent the living population, the results might not accurately reflect the true (sero) prevalence. Furthermore, PyV VP1 VLPs are known to have a relatively high degree of cross-reactivity [80], thus it is possible that seroreactivity is showing indirectly the presence of another PyV in the sample. However, since there is no other PyV reported in garden dormice so far, and another edible dormouse infecting PyV is a representative of distant *Betapolyomavirus* genus [17], we believe that the seroprevalence data presented in this study should correspond to the virus whose DNA was identified.

Interestingly, PyV DNA prevalence was higher than seroprevalence (36% for BVPyV and 13% for CVPyV) in the forementioned vole study [24], which is the opposite result to ours. In our case, PyV DNA was detected in only two animals (2.7%, 95% CI: 0-6.4%), both of which were seronegative. Such differences might be related to lifespan or seasonal behavior of these animals. Puumala hantavirus seroprevalence in bank voles has been shown to vary significantly across different seasons and years [82], and the detection rates of *Borrelia* spp. DNA fluctuates in edible dormice due to hibernation cycles and reduced pathogen exposure during inactive periods [83]. However, PyVs are known to cause persistent infection, thus identification of PyV DNA in infected tissues is expected. It raises the question of whether the garden dormouse is the reservoir for this PyV, as one would expect to find some animals that are both DNA- and antibody-positive, rather than just those with acute infections (DNA only) or those that have cleared the infection (antibodies only). PyVs, such as human HPyV1, HPyV2, and mouse MPyV1, were shown to be persisting in kidneys, however, PyV DNA is not homogenously spread across the whole organ of a healthy individual, causing false-negative detection results [84]. Kidneys also seem to be a good target for the search of novel rodent PyVs, since in most studies where these PyVs were identified, PyV DNA was detected either in kidney samples, or in other tissues as well as kidneys (if kidney samples were available and tested) [12, 17, 20, 22, 24]. Yet, it is not clear if dormouse PyV preferentially infects the kidneys as primary tissue for infection and persistence, since no other tissues were tested in this study. Further investigations would benefit from testing additional tissues, especially

liver in relation to the preliminary pathology observed. The use of late PyV genomic region targeting primers in parallel to LTAg diagnostic primers could also be considered for an improved sensitivity. Considering the endangered status of the species, non-invasive methods, such as swabs, feces and urine collection, could be implemented for PyV infection monitoring [13, 28].

Since novel dormouse PyV LTAg coding sequence diverges from its closest relatives *Alphapolyomavirus quartipanos* and *Alphapolyomavirus quintipanos* by over 30%, following the species definition criteria of the ICTV (required minimal divergence from the closest PyV LTAg coding sequence – 15%) this could be considered a novel member of the genus *Alphapolyomavirus*. We consider that this is a new garden dormouse-infecting PyV and suggest tentatively naming it Eliomys quercinus polyomavirus 1 (EquePyV1), tentative species name *Alphapolyomavirus equercinus.*

Conclusions

Here we describe a novel polyomavirus in the garden dormouse with molecular and serological detection. The pairwise sequence comparison and phylogenetic analysis suggest that this virus may represent a novel species within the genus *Alphapolyomavirus*. Future studies are needed to confirm the host association of this novel virus and to address the question of whether the virus is associated with disease in dormice or directly contributing to the population decline of these animals.

Abbreviations

aa	Amino acid
ALTO	Alternative T Open Reading Frame
BVPyV	Bank Vole (*Clethrionomys glareolus*) Polyomavirus, *Betapolyomavirus myoglareolus*
CCF	Chest Cavity Fluid
CR1	Conserved Region 1
CVPyV	Common Vole (*Microtus arvalis*) Polyomavirus, *Betapolyomavirus marvalis*
DB450/150	yeast Disruption Buffer with 450/150 mM NaCl
HaPyV	Syrian hamster (*Mesocricetus auratus*) polyomavirus, *Alphapolyomavirus mauratus*
HPyV(s)1–16	Human Polyomavirus(es) 1–16
Hsc70	Heat Shock Cognate 70
ICTV	International Committee on Taxonomy of Viruses
IUCN	International Union for Conservation of Nature
LTAg, MTAg, sTAg	polyomavirus tumor antigens – Large T (LTAg), small T (sTAg) and Middle T (MTAg)
MYA	million years ago
NCBI	National Center for Biotechnology Information
NLS	Nuclear Localization signals
NTAR	N-Terminal Activating Region
ORF	Open Reading Frame
PI3K	Phosphatidylinositol 3-kinase
PLCγ1	Phospholipase C gamma 1
PP2A	Phosphatase 2 A
pRb	Retinoblastoma protein
Pur-alpha	Purine-rich element binding protein A
PyV(s)	Polyomavirus(es)
SH3	Src Homology 3
ShcA	Src homology 2 domain containing protein A
SRA	Sequence Read Archive
SV40	Simian Virus 40; macaque polyomavirus, *Betapolyomavirus macacae*
VLPs	Virus-Like Particles
VP1,2,3	Viral Proteins 1, 2, 3

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions

E.V., A.G., and R.G.U. conceptualized and designed the study. E.M.K., J.L., C.J., and R.G.U. acquired the dormice samples. E.V. and M.R. conducted the experiments. E.V. performed the data analysis and prepared the initial manuscript draft. R.G.U., C.B.B., and A.G. provided expertise on data analysis and interpretation. All authors contributed to the manuscript revision and approved the final version for submission.

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Data availability

Sequenced genomes were submitted to the National Center for Biotechnology Information (PQ246041 and PQ246042). Other additional data is provided in the Supplementary files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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