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Lymphocryptovirus phylogeny and the origins of Epstein–Barr virus

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Specimens from wild and captive primates were collected and novel members of the genus *Lymphocryptovirus* (subfamily *Gammaherpesvirinae*) were searched for utilizing PCR for the DNA polymerase gene. Twenty-one novel viruses were detected. Together with previous findings, more than 50 distinct lymphocryptoviruses (LCVs) are now known, with hosts from six primate families (Hominidae, Hylobatidae, Cercopithecidae, Atelidae, Cebidae and Pitheciidae). Further work extended genomic sequences for 25 LCVs to 3.4–7.4 kbp. Phylogenetic trees were constructed, based on alignments of protein sequences inferred from the LCV genomic data. The LCVs fell into three major clades: Clade A, comprising New World viruses; Clade B, containing both Old World monkey viruses and hominoid viruses including Epstein–Barr virus (EBV); and Clade C, containing other hominoid viruses. By comparison with the primate tree, it was proposed that major elements of the LCV tree represented synchronous evolution with host lineages, with the earliest node in both trees being the separation of Old and New World lines, but that some virus lineages originated by interspecies transfer. From comparisons of branch lengths, it was inferred that evolutionary substitution in Clade B has proceeded more slowly than elsewhere in the LCV tree. It was estimated that in Clade B a subclade containing EBV, a gorilla virus and two chimpanzee viruses derived from an Old World monkey LCV line approximately 12 million years ago, and another subclade containing an orang-utan virus and a gibbon virus derived from a macaque LCV line approximately 1.2 million years ago.

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INTRODUCTION

This paper is concerned with relationships among viruses of the genus *Lymphocryptovirus* (subfamily *Gammaherpesvirinae*, family *Herpesviridae*, order *Herpesvirales*; Davison *et al.*, 2009). Lymphocryptoviruses (LCVs) all have primate hosts, and Epstein–Barr virus (EBV) is the single known human LCV. Historically, the subfamily *Gammaherpesvirinae* was treated as consisting of the gamma-1 and gamma-2 groups. The term gamma-1 group is now superseded in formal taxonomy by the genus *Lymphocryptovirus*. Three genera are presently assigned to the gamma-2 group (*Rhadinovirus*, *Percavirus* and *Macavirus*) and there are additional gamma-2 lineages for which the taxonomy has not yet been developed (Davison *et al.*, 2009; Ehlers *et al.*, 2008). Phylogenetic trees for the family *Herpesviridae*, based on molecular sequences, display large-scale features within each of the three subfamilies that have been interpreted as showing synchronous development of major viral lineages with the lineages of the mammalian hosts, and such cospeciation has evidently been a prominent mode in the evolution of this virus family (McGeoch *et al.*, 2006, 2008). It is considered that in the subfamily *Gammaherpesvirinae* the gamma-1 branch originated cospeciationally with the primate lineage (McGeoch *et al.*, 2006).

EBV was the first gammaherpesvirus identified (Epstein *et al.*, 1964) and it was later classified as the type species of the genus *Lymphocryptovirus*. EBV causes infectious

Supplementary tables are available with the online version of this paper.

mononucleosis and is associated with various tumours in humans (Pagano, 1999). In Old World non-human primates, evidence for EBV-like LCVs was initially obtained by serological cross-reactivity to EBV, in chimpanzees (Landon *et al.*, 1968), orang-utans (Rasheed *et al.*, 1977), gorillas (Neubauer *et al.*, 1979), baboons (Vasiljeva *et al.*, 1974) and diverse macaque species (Fujimoto *et al.*, 1990; Hayashi *et al.*, 1999; Rangan *et al.*, 1986; Rivadeneira *et al.*, 1999). More recently, PCR-based methods have been used to detect LCVs in Old World and New World primates (Ramer *et al.*, 2000; Cho *et al.*, 2001; Ehlers *et al.*, 2003; Prepens *et al.*, 2007), and to date about 30 different LCVs are known.

In a previous study, we amplified partial sequences for DNA polymerase (DPOL; BALF5 in EBV) genes of 26 novel LCVs and analysed them phylogenetically. Three major clusters of LCVs were separated in the phylogenetic tree, one comprising LCVs of New World monkeys, the other two comprising LCVs of hominoids. One of them (Genogroup 1) was highly populated and contained, besides hominoid LCVs, including EBV, those of several Old World monkeys. The other group (Genogroup 2) was small and contained viruses of apes, but no monkey LCVs. Gorilla viruses were present in both groups. From these findings (in particular the last), we proposed that two lineages of Old World primate LCVs might exist, each with representatives in most primate species (Ehlers *et al.*, 2003).

In the present study, we followed two experimental lines to reassess this hypothesis. First, we searched for additional LCVs of Genogroup 2, particularly in chimpanzees and Old World monkeys. Second, we set out to extend the short DPOL sequences, which were the only available data for most of the described LCVs, by a bigenic PCR approach also targeting the glycoprotein B (gB; BALF4 in EBV) gene. For a subset of these viruses, we determined sequences that spanned up to four genes. We have performed phylogenetic analyses with this much larger dataset and have revised our interpretation of LCV evolution.

RESULTS

Multigenic *de novo* detection of novel LCV

Over six years, blood, tissue and faeces samples (n=502) were collected from live or deceased individuals of 49 primate species (apes, Old World monkeys, New World monkeys and prosimians, Table 1) as described in Methods. LCV DPOL gene sequences were successfully amplified from 50 % of the samples (n=251) with panherpes DPOL PCR. Sequences identical to some of these were detected previously (Ehlers *et al.*, 2003), but most came from 21 novel putative LCVs. They are listed with their names, abbreviations, hosts and GenBank accession numbers in Table 2. Combined with the 26 species detected previously (Ehlers *et al.*, 2003), a total of 47 LCVs were discovered. The LCV-positive primate hosts originated from seven African, Asian and European countries, and were members of six different primate families, three of the Catarrhini (Hominidae, Hylobatidae and Cercopithecidae) and three of the Platyrrhini (Atelidae, Cebidae and Pitheciidae). No prosimian LCVs were detected.

For amplification of the major DNA-binding protein (MDBP; BALF2 in EBV) gene and gB gene sequences, nested degenerate, deoxyinosine-substituted (deg/dI) primer sets (BALF2A and BALF4A, respectively) were derived from known LCV sequences (CalHV-3 BALF2 and PtroLCV-1 BALF4) with the primer-binding sites placed within regions of high gammaherpesvirus conservation. We amplified MDBP and gB gene sequences from most of the 47 LCV species with BALF2A and BALF4A primer sets. Amplification of GgorLCV-2 and SsynLCV-1 with BALF2A and BALF4A yielded incorrect sequences or failed, since many GgorLCV-2 and all SsynLCV-1-positive samples were double-infected with GgorLCV-1 and SsynLCV-2, respectively. We therefore used PpygLCV-1 sequences – once determined – to design the alternative primer sets BALF2B and BALF4B. For 25 LCVs, the gB sequences could be connected to the corresponding DPOL sequences with Long-Distance (LD)-PCR using virus-specific gB-sense primers and DPOL-antisense primers. Contiguous sequences of 3.4 kbp spanning the 3′-part of the gB gene and the 5′-part of the DPOL gene were obtained (Table 2). For 11 of 25 LCVs, MDBP sequences could be connected to gB with a second LD-PCR, and contiguous sequences of up to 7.5 kbp were determined, spanning two-thirds of the DPOL gene and the complete gB gene, and in some cases also the complete BALF3 gene (Fig. 1).

Preliminary phylogenetic analysis showed that, of the novel LCVs found in the present study, only one belonged to Genogroup 2 (HmueLCV-1), with all the others in either Genogroup 1 or a clade of New World monkey viruses. To search further for LCVs belonging to Genogroup 2, we designed a set of nested deg/dI primers based on the gB gene of GgorLCV-2 and therefore biased towards the detection of Genogroup 2 LCVs (primer set BALF4C); with this set, we tested samples of chimpanzees and macaques, with gorilla samples as controls. Variants of the gB genes of GgorLCV-2 and PtroLCV-1 were detected, but no chimpanzee or macaque LCVs were detected as members of Genogroup 2. In addition, we designed a set of nested deg/dI primers based on the DPOL gene of GgorLCV-2 with a 3′-base that was present in all DPOL gene sequences of Genogroup 2, but not of Genogroup 1 (primers not listed); with this set, the LCVs of Genogroup 2 were detected in gorillas, orang-utans and gibbons, but chimpanzee and macaque specimens tested negative.

Phylogenetic relationships in the genus *Lymphocryptovirus*

The 25 LCV gB to DPOL sequences determined in this study plus the homologous sequences of EBV, CalHV-3 and CeHV-15 were subjected to phylogenetic analysis.

Table 1. Primate hosts of LCVs

Host family, subfamily, genus	Host species	
Catarrhini (Old World monkeys and apes)		
Family Hominidae		
Genus *Gorilla*	*Gorilla gorilla*	Gorilla
Genus *Pan*	*Pan troglodytes*	Chimpanzee
	Pan paniscus	Bonobo
Genus *Pongo*	*Pongo pygmaeus*	Borneo orang-utan
Family Hylobatidae		
Genus *Hylobates*	*Hylobates lar*	White-handed gibbon
	Hylobates muelleri	Mueller's gibbon
Genus *Symphalangus*	*Symphalangus syndactylus*	Siamang
Family Cercopithecidae		
Subfamily Cercopithecinae		
Genus *Cercocebus*	*Cercocebus atys*	Sooty mangabey
Genus *Cercopithecus*	*Cercopithecus hamlyni*	Owl-faced monkey
	Cercopithecus cephus	Moustached guenon
	Cercopithecus neglectus	De Brazza's monkey
	Cercopithecus nictitans	Greater spot-nosed guenon
Genus *Chlorocebus*	*Chlorocebus aethiops*	Vervet monkey
Genus *Erythrocebus*	*Erythrocebus patas*	Patas monkey
Genus *Lophocebus*	*Lophocebus albigena*	Grey-cheeked mangabey
	Lophocebus aterrimus	Black mangabey
Genus *Macaca*	*Macaca fascicularis*	Long-tailed macaque
	Macaca thibetana	Tibetan stump-tailed macaque
	Macaca fuscata	Japanese macaque
	Macaca mulatta	Rhesus monkey
Genus *Mandrillus*	*Mandrillus sphinx*	Mandrill
Genus *Miopithecus*	*Miopithecus talapoin*	Dwarf guenon
Genus *Papio*	*Papio anubis*	Olive baboon
	Papio hamadryas	Hamadryas baboon
Subfamily Colobinae		
Genus *Colobus*	*Colobus guereza*	Black-and-white colobus
	Colobus polykomos	King colobus
Genus *Piliocolobus*	*Piliocolobus badius*	Red colobus
Genus *Semnopithecus*	*Semnopithecus entellus*	Hanuman langur
Platyrrhini (New World monkeys)		
Family Atelidae	*Ateles paniscus*	Black spider monkey
Family Cebidae	*Callithrix penicillata*	Black-pencilled marmoset
	Callithrix jacchus	Common marmoset
	Leontopithecus rosalia	Golden lion tamarin
	Saimiri sciureus	Common squirrel monkey
Family Pitheciidae	*Pithecia pithecia*	White-faced saki

First, a phylogenetic tree based on gB sequences of 58 viruses from all three subfamilies of the *Herpesviridae* was constructed, and is shown in summary form in Fig. 2(a), to provide an overview of the context of the gamma-1 lineage. This tree shows that all the LCVs fall into three major clades (designated A, B and C), and that the gamma-2 group can be utilized as an outgroup of related species to place the root of the gamma-1 tree. A tree for the BALF3 gene computed with Bayesian analysis utilizing Monte Carlo Markov chains (BMCMC), and based on an alignment of 634 aa residues for 12 LCVs, displayed the same three clades (not shown). Fig. 2(b) shows a detailed gamma-1 tree, based on a 946 residue alignment of gB and DPOL amino acid sequences for 28 LCVs plus 11 gamma-2 primate viruses, and derived by BMCMC. The data for this tree represent the largest set of sequences and longest alignment available to give a robustly rooted gamma-1 phylogeny. The three major clades and their contents are labelled. Clade A contains all the LCVs of New World monkeys and no other viruses. Clade B contains all the LCVs of Old World monkeys plus some viruses of hominoids, including EBV. Clade C contains only hominoid viruses. Clades B and C correspond to Genogroups 1 and 2, respectively. On the basis of the clade contents and branching pattern we hypothesized that, at the level of these major clades, the tree structure

reflects synchronicity with that of the host lineages, in that divergence of New World and Old World lineages was the earliest branching event for both hosts and viruses, followed by divergence of Old World monkey and hominoid lineages. This interpretation is straightforward for Clades A and C, corresponding to New World monkey and hominoid hosts, respectively, while Clade B was taken to correspond to Old World monkey hosts, leaving aside at this point the issue of hominoid viruses in Clade B.

The extent of correspondences between host and LCV lineages was then examined in detail. Fig. 3(a) displays a primate phylogenetic tree containing host species for all the LCVs whose sequences were included in our analyses and Fig. 3(b) shows a molecular clock version of the LCV tree of Fig. 2(b). The data for Fig. 3(a) were extracted from the large study of mammalian phylogeny of Bininda-Emonds *et al.* (2007), and the figure shows the timescale derived by those authors. The two trees in Fig. 3 were drawn to have the same size across the page from branch tips to root in order to facilitate comparisons between them, taking divergence of Old and New World primate LCV lineages as synchronous with divergence of the equivalent host lineages. In Clade C the arrangement of branches for LCVs of gibbon species, orang-utan (*Pongo pygmaeus*) and gorilla (*Gorilla gorilla*) matches with the host tree, as do the depths of branches except for those of the two gibbon viruses. In Clade A there are four deep branches, three of which match arrangement and approximate depth with the host tree, while that for CalHV-3 and CpenLCV-1 is incongruent with the corresponding host locus. Overall, then, Clades A and C, together with the deeper portion of the LCV tree, present as mostly consistent with cospeciational development in terms of patterns and proportions of branches.

Clade B is more complicated, in several respects: it is the most populous of the three major clades; the overall depth of branch lengths is less than in Clades A and C; some loci are poorly or incompletely resolved; and hominoid viruses appear at two loci among the majority Old World monkey viruses. In all, Clade B presented the main challenges for interpretation of the LCV tree. A tree specific to the 28 LCVs was therefore constructed, to provide the best achievable resolution of Clade B with the available data. This utilized a 1042 residue alignment of gB and DPOL amino acid sequences and was inferred by BMCMC. Fig. 4(a) shows only Clade B from this tree, with the root position provided by Clades A and C, and with branch lengths drawn at a larger scale than in the earlier figures. Four nodes in the tree presented in Fig. 4(a) have low posterior probabilities, and these were reduced to multifurcations before computing a molecular clock version. Two additional LCVs (CateLCV-1 and SsynLCV-2), for which less extensive sequence data were available, were then interpolated into the molecular clock tree, as shown in Fig. 4(b). For HmueLCV-1 (Clade C) and 17 Clade B viruses only short sequences of 175–430 bp were available, and these were not included in the molecular clock tree. For discussion, seven clades comprising the tree were designated B1–B7, and the multifurcated node from which five of these clades descend was designated the 'major multifurcation' (MMF).

Considering only the monkey viruses in Clade B in the first instance, some aspects of the branching pattern can be seen to correspond with that of the host tree, while others do not. Thus, the branching relationships among SentLCV-1, EpatLCV-1, the two PhamLCVs (subclade B5) and macaque LCVs in subclade B6 are congruent with the host tree. Short branch lengths and branching uncertainties among the macaque LCVs limit the detail of comparisons for this grouping. The loci of MsphLCV-1, CgueLCV-1, PbadLCV-1 and CnegLCV-1 do not fit into this cospeciational scheme. Turning to the six hominoid viruses in Clade B, we note that these occur at two distinct loci. Subclade B3 (comprising EBV, GgorLCV-1, PpanLCV-1 and PtroLCV-1) originates in the midst of the monkey LCV lineages, and the branching pattern within this grouping is partly, but not completely, compatible with cospeciational development (in agreement with the earlier observations of Gerner *et al.*, 2004). PpygLCV-2 and SsynLCV-2 appear together within the predominantly macaque virus B6 subclade, and are readily rationalized as late transfers from a macaque host.

With respect to the possible Old World monkey cospeciational components of Clade B noted above, the branch lengths in Figs 3(b) and 4(b) are markedly shorter than would be expected from comparison with those in Fig. 3 of Clades A and C, and of the host tree, so that a cospeciational rationale for Clade B would imply that lineages in that clade have been changing more slowly than those in Clades A and C. The most straightforward way to assign a local cospeciational timeframe for Clade B is to take the node at which the SentLCV-1 lineage (B1 subclade) diverges from the other subclades as corresponding to that in the host tree (Fig. 3a) at which the lineage of the host species for SentLCV-1, *Semnopithecus entellus*, diverges from lineages leading to *Macaca*, *Papio*, etc. These nodes are the earliest in Clade B and in the Old World monkey clade, respectively. The timescale shown in Fig. 4(b) is based on this assignment, and represents a relative substitution rate of 0.6 times that is proposed for the whole gamma-1 tree in Fig. 3(b).

The MMF node is a central feature of the tree in Fig. 4(b), and was also observed in trees based on subsets of the available amino acid sequences and in trees for Old World primate LCVs based on DNA sequences (not shown). We take the MMF feature to be a result of the available sequence data being inadequate to resolve several closely spaced nodes. We note that the posterior probability support for subclade B2 branching from an earlier node than the MMF, as presented in Fig. 4(b), is marginal. If we consider just the monkey virus lineages descending from the MMF, evidently both the *Papio* LCV line (B5) and the

Table 2. Viruses, abbreviations and GenBank accession numbers

Host species	Origin of host	Virus name	Abbreviation	Novel virus*	gB-DPOL sequence†	GenBank accession number
Viruses from this study						
Old World primates						
Sooty mangabey	Ivory coast	*Cercocebus atys* lymphocryptovirus 1	CatyLCV-1	x		GQ921921
Owl-faced monkey	Germany (zool. gardens)	*Cercopithecus hamlyni* lymphocryptovirus 1	ChamLCV-1	x		AY608706
Moustached guenon	Cameroon	*Cercopithecus cephus* lymphocryptovirus 1	CcepLCV-1	x		AY608711
De Brazza's monkey	Cameroon	*Cercopithecus neglectus* lymphocryptovirus 1	CnegLCV-1	x	x	AY728176
De Brazza's monkey	Cameroon	*Cercopithecus neglectus* lymphocryptovirus 2	CnegLCV-2	x		AY608712
Greater spot-nosed guenon	Cameroon	*Cercopithecus nictitans* lymphocryptovirus 1	CnicLCV-1	x		AY608709
Vervet monkey	Germany (primate facility)	*Chlorocebus aethiops* lymphocryptovirus 1	CaetLCV-1	x		AY608702
Vervet monkey	Germany	*Chlorocebus aethiops* lymphocryptovirus 2	CaetLCV-2	x		GQ921922
Black-and-white colobus	Cameroon; Germany (zool. gardens; primate facility)	*Colobus guereza* lymphocryptovirus 1	CgueLCV-1		x	AF534219
King colobus	Ivory coast	*Colobus polykomos* lymphocryptovirus 1	CpolLCV-1	x		GQ921923
Patas monkey	Cameroon; Germany (primate facility)	*Erythrocebus patas* lymphocryptovirus 1	EpatLCV-1		x	AY196148
Gorilla	Congo; Cameroon; Germany, Belgium, USA (zool. gardens); cell line	*Gorilla gorilla* lymphocryptovirus 1	GgorLCV-1		x	AF534225
Gorilla	Congo; Cameroon; Germany (zool. gardens)	*Gorilla gorilla* lymphocryptovirus 2	GgorLCV-2		x	AY129395
White-handed gibbon	Germany (zool. gardens)	*Hylobates lar* lymphocryptovirus 1	HlarLCV-1		x	AY196147
Mueller's gibbon	Germany (zool. gardens)	*Hylobates muelleri* lymphocryptovirus 1	HmueLCV-1	x		AY273184
Grey-cheeked mangabey	Cameroon	*Lophocebus albigena* lymphocryptovirus 1	LalbLCV-1	x		AY608710
Black mangabey	Germany (private husbandry)	*Lophocebus aterrimus* lymphocryptovirus 1	LateLCV-1		x	AY174067
Long-tailed macaque	Germany (primate facility, zool. gardens)	*Macaca fascicularis* lymphocryptovirus 1	MfasLCV-1		x	AF534221
Japanese macaque	Germany (primate facility)	*Macaca fuscata* lymphocryptovirus 1	MfusLCV-1		x	AF534224
Japanese macaque	Germany (primate facility)	*Macaca fuscata* lymphocryptovirus 2	MfusLCV-2		x	AY172954
Tibetan stump-tailed macaque	Germany (primate facility)	*Macaca tibetana* lymphocryptovirus 2	MtibLCV-2	x	x	GQ921925
Mandrill	Germany (primate facility, zool. gardens)	*Mandrillus sphinx* lymphocryptovirus 1	MsphLCV-1		x	AF534227
Mandrill	Cameroon; Germany (zool. gardens)	*Mandrillus sphinx* lymphocryptovirus 2	MsphLCV-2	x		AY728172
Dwarf guenon	Cameroon	*Miopithecus talapoin* lymphocryptovirus 1	MtalLCV-1	x		AY608708
Bonobo	South Africa; Germany (zool. gardens)	*Pan paniscus* lymphocryptovirus 1	PpanLCV-1		x	AF534220

Table 2. cont.

Host species	Origin of host	Virus name	Abbreviation	Novel virus*	gB-DPOL sequence†	GenBank accession number
Chimpanzee	Ivory Coast; Uganda; Congo; South Africa; Germany (primate facility; zool. gardens); cell line	*Pan troglodytes* lymphocryptovirus 1	PtroLCV-1		x	AF534226
Olive baboon	Cameroon; Tanzania	*Papio anubis* lymphocryptovirus 1	PanuLCV-1	x		AY728174
Hamadryas baboon	Tanzania; Germany (zool. gardens)	*Papio hamadryas* lymphocryptovirus 2	PhamLCV-2		x	AF534229
Red colobus	Ivory coast	*Piliocolobus badius* lymphocryptovirus 1	PbadLCV-1		x	AF534228
Red colobus	Democratic Republic Congo	*Piliocolobus badius* lymphocryptovirus 2	PbadLCV-2	x		GQ921927
Orang-utan	Indonesia; Germany (zool. gardens)	*Pongo pygmaeus* lymphocryptovirus 1	PpygLCV-1		x	AY129398
Orang-utan	Germany (zool. gardens)	*Pongo pygmaeus* lymphocryptovirus 2	PpygLCV-2	x	x	GQ921926
Hanuman langur	Germany (zool. gardens)	*Semnopithecus entellus* lymphocryptovirus 1	SentLCV-1		x	AF534223
Siamang	Germany (primate facility)	*Symphalangus syndactylus* lymphocryptovirus 1	SsynLCV-1		x	AY608703
Siamang	Germany (primate facility)	*Symphalangus syndactylus* lymphocryptovirus 2	SsynLCV-2	x	x	GQ921924
New World primates						
Black spider monkey	Germany (zool. gardens)	*Ateles paniscus* lymphocryptovirus 1	ApanLCV-1		x	AY139028
Black-pencilled marmoset	Germany (primate facility)	*Callithrix penicillata* lymphocryptovirus 1	CpenLCV-1		x	AY139026
Golden-lion tamarin	Germany (primate facility)	*Leontopithecus rosalia* lymphocryptovirus 1	LrosLCV-1	x		AY608705
White-faced saki	Germany (zool. gardens)	*Pithecia pithecia* lymphocryptovirus 1	PpitLCV-1		x	AY139025
Common squirrel monkey	French Guinea; Germany (zool. gardens)	*Saimiri sciureus* lymphocryptovirus 2	SsciLCV-2		x	AY139024
Common squirrel monkey	Germany (zool. gardens)	*Saimiri sciureus* lymphocryptovirus 3	SsciLCV-3	x		AY854172
Published viruses						
Complete genome						
Human		Epstein–Barr virus	EBV=HHV-4			NC_007605
Rhesus monkey (*Macaca mulatta*)		Rhesus monkey lymphocryptovirus	RLV=CeHV-15			AY037858
Marmoset (*Callithrix jacchus*)		Callitrichine herpesvirus 3	CalHV-3			AF319782
gB-DPOL sequence						
Hamadryas baboon	Tanzania; Germany	*Papio hamadryas* lymphocryptovirus 3	PhamLCV-3			EU118146

*Viruses that were discovered in the course of the present study are marked.
†Viruses for which we determined contiguous gB plus DPOL sequences are marked.

Macaca LCV line (in B6) are in cospeciation-compatible loci, and the locus of the CateLCV-1 line (B7) could result from lack of resolution for placing that virus with the *Papio* viruses as the host species are in Fig. 3(a).

Of the lineages originating directly from the MMF, this leaves only the hominid virus lineage B3 and the PbadLCV-1 line (B4) requiring transfer between host species to account for their placing. The Clade B local timescale of

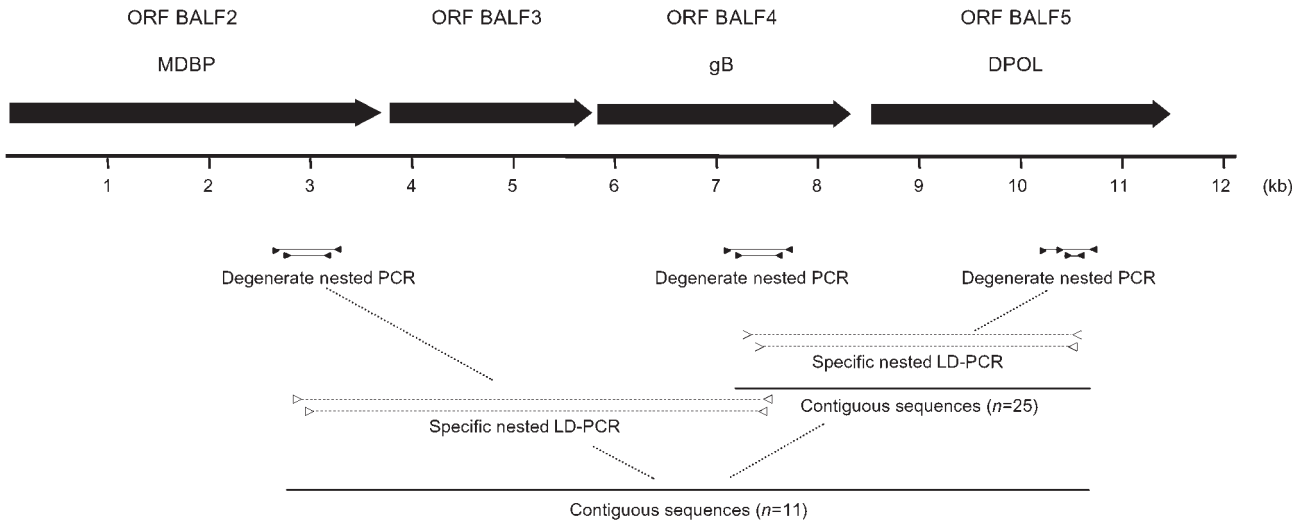

Fig. 1. Map of amplified genes and diagrams of PCR strategies. Degenerate nested primers were used to amplify part of the MDBP, the gB or the DPOL genes. The amplified fragments are represented by thin solid lines between the primer-binding sites (black triangles). LD nested PCR was performed with specific primers. The amplified fragments are represented by dashed lines between the primer-binding sites (open triangles). The numbers of gB to DPOL and MDBP to DPOL sequences are specified, and their locations are depicted with thick solid lines. At the top of the figure, the genomic locus spanning ORF BALF2 (MDBP) to ORF BALF5 (DPOL) is depicted with black arrows. The arrowhead indicates the direction of transcription. The ORF designation is adapted from the ORF nomenclature of EBV. The start of the ruler corresponds with the first base of the ORF BALF2.

Fig. 4(b) dates the MMF node to about 12 millions of years (MYA) before present, and we thus interpret these features to indicate that the hominid LCV lineage B3 arose from a monkey LCV lineage within the last 12 million years. Because the branching order of the hominid LCVs in B3 does not match that of their hosts (Fig. 3a), development of B3 must have involved minimally two transfer events: one from a monkey to a hominid host, and the other either also from a monkey to a hominid host or between distinct hominid lineages. The precise origin of the B3 lineage is obscured by the MMF, but in principle the line could have arisen from the single lineage immediately ancestral to other, unresolved branchings covered by the MMF, or from within unresolved branchings of the MMF, or from an early point in a monkey LCV lineage descendant from the MMF (not necessarily one of those visible as B4–B7). The first of these scenarios is weakly supported in the tree of Fig. 4(a). Turning to subclade B6 and the three non-macaque viruses therein (CnegLCV-1, PpygLCV-2 and SsynLCV-2), these all lie in one tightly delimited clade with MfasLCV-1. Notably, MfasLCV-1 and the two hominoid viruses all have hosts from South-East Asia, but the host of CnegLCV-1 is African. The multifurcated node giving rise to these viruses has an estimated date of 1.2 MYA.

DISCUSSION

Two experimental lines were followed in the present study to improve our understanding of LCV evolution. First, we searched for additional viruses belonging to Genogroup 2. In this study, >500 specimens were collected from live or deceased individuals of 49 primate species (hominoids, Old World monkeys, New World monkeys, prosimians) from three continents. In a previous survey (Ehlers *et al.*, 2003), >600 specimens were analysed. From both studies, covering a total of >1100 specimens, sequences were detected from 47 distinct novel LCVs. Among these were no LCVs of chimpanzees or Old World monkeys in Clade C. We have also searched for possible human LCVs in Clade C but found only known variants of EBV, belonging to Clade B (data not shown). Based on these combined data, we conclude that the existence of two distinct and complete Old World primate LCV lineages as previously proposed (Ehlers *et al.*, 2003) is less likely. In a second step, we extended the amounts of sequence information for LCVs by up to sevenfold. This was successful for 25 viruses but failed for the remainder, probably because of genome copy numbers being too low for LD-PCR. With this much larger dataset we re-examined LCV phylogeny, extending and refining the phylogenetic analysis published earlier (Ehlers *et al.*, 2003). In particular, the LCV phylogeny could now be compared with host phylogeny with adequate precision, and the topology of Clade B could be analysed with higher resolution.

Interpretation of the genus *Lymphocryptovirus* tree as reflecting long-term synchronous development with primate host lineages accounts well for the pattern of major branches, and is in harmony with features in other

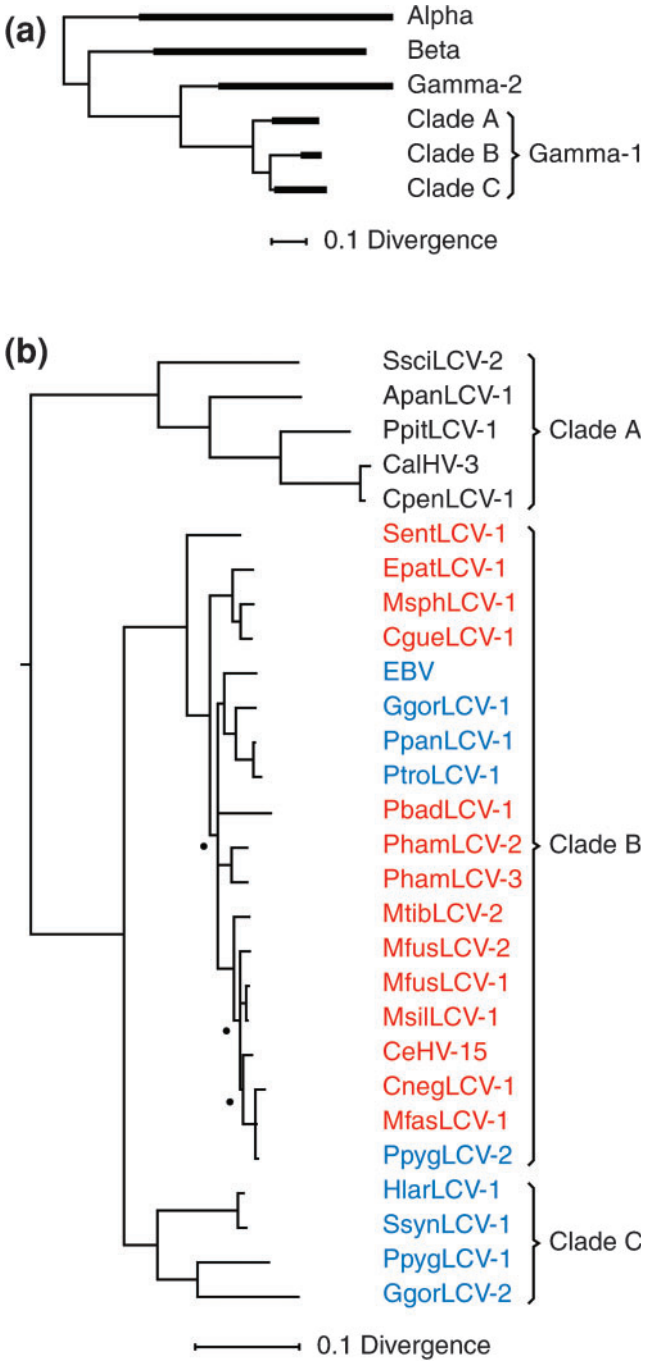

Fig. 2. Phylogenetic trees for the family *Herpesviridae* and the genus *Lymphocryptovirus*. (a) Overview tree based on a 641 residue alignment of gB amino acid sequences for 58 herpesviruses (listed in Supplementary Table S2, available in JGV Online). The tree was obtained by the neighbour-joining method and is midpoint rooted. The *Alphaherpesvirinae* (Alpha) and *Betaherpesvirinae* (Beta) branches are shown in summary form, with regions containing multiple branches reduced to single heavy lines. In the subfamily *Gammaherpesvirinae*, the gamma-2 subgroup is also shown in summary form, and for the gamma-1 subgroup the three major clades (A, B and C) are depicted. (b) BMCMC tree for the genus *Lymphocryptovirus*. A 946 residue alignment of concatenated partial gB and DPOL amino acid sequences for 28 LCVs plus 11 gamma-2 primate herpesviruses was constructed and analysed by BMCMC. The gamma-2 sequences (listed in Supplementary Table S3, available in JGV Online) served as an outgroup to locate the root for the LCV clade, and are not shown in the figure. The LCV tree is shown as a majority rule consensus tree. Branch labels for LCVs with New World monkey hosts are in black, for those with Old World monkey hosts are in red, and for those with ape or human hosts are in blue. The major clades (A, B and C) are labelled. There are three multifurcations in Clade B, which represent loci that were not resolved by the BMCMC process. All resolved nodes have posterior probability of 1.00, except for the three marked with filled black circles, which have posterior probabilities in the range 0.71–0.78. A scale indicating divergence, as substitutions per site, is at the foot.

parts of the family *Herpesviridae* tree (McGeoch *et al.*, 2006, 2008). However, to account for aspects of relative branch lengths, it is then necessary to propose differing rates of evolutionary change for different regions within the genus *Lymphocryptovirus* tree. In this connection, we note that phylogenetic analyses typically show a markedly lower rate of change in the whole gamma-1 lineage than in the gamma-2 lineage (for instance, see branch lengths in Fig. 2a; also, McGeoch *et al.*, 2006, 2008). We modelled the rate difference proposed between Clade B and other parts of the genus *Lymphocryptovirus* tree by applying a single decreased rate from the earliest node in Clade B. We regard this as a simple and justified device, while emphasizing that its ad hoc nature urges caution in applying the resulting Clade B timeframe. While we have no data on the underlying causes of decreased rates of change, both in gamma-1 lineage relative to gamma-2 and in Clade B relative to other parts of gamma-1, it is interesting to speculate: perhaps these phenomena reflect stages in elaboration of the mode of virus existence and latency exemplified by EBV (Young & Rickinson, 2004).

In summary, elements of both cospeciation and horizontal transmission were observed in LCV evolution with the present study. From the perspective of human virology, it is of interest that EBV belongs to a lineage that arose by interspecies transfer from a line of Old World monkey viruses. However, transfer events from monkey to hominid host lineages appear to belong to an era that lies far back in the evolutionary development of humans and great apes: EBV could not be regarded as an agent that is novel to its present day host species. On our interpretation, Clade C (Genogroup 2) contains hominoid virus lines that have developed in long-term synchrony with evolution of their host species. Surprisingly, we have not detected any chimpanzee viruses in this group, and neither is any human virus known. No obvious reason is apparent for

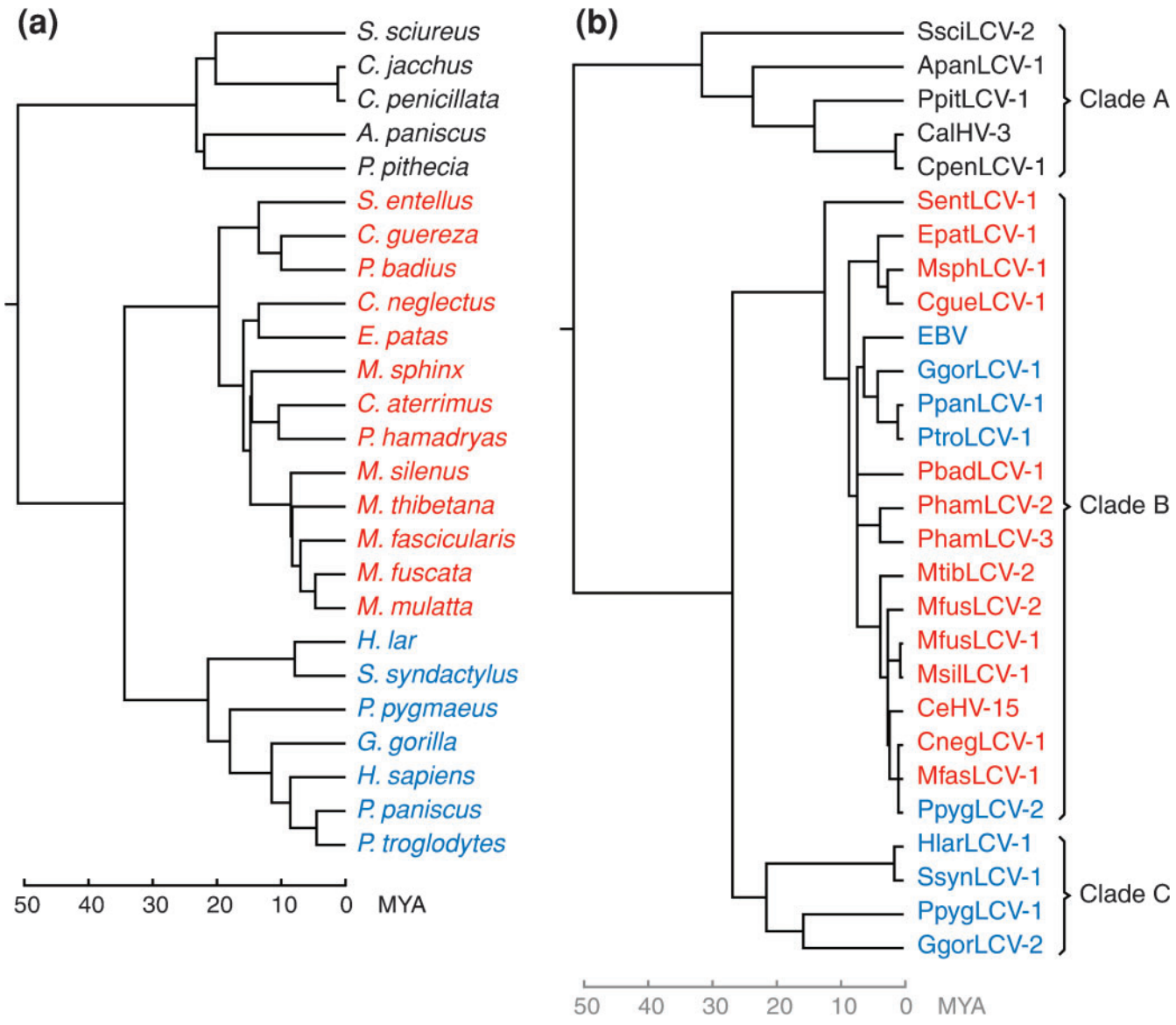

Fig. 3. Comparison of phylogenetic trees for primate hosts and LCVs. (a) Tree for primate species that appear as hosts of LCVs in this paper. Data for this tree were extracted from the study of Bininda-Emonds *et al.* (2007). New World monkey species are listed in black, Old World monkey species in red, and apes plus humans in blue (*Cercocebus aterrimus* does not feature as a host until Fig. 4b). A timescale is shown at the foot, as millions of years (MYA) before present. (b) Molecular clock tree for LCVs. The tree shown was computed from the 39 LCVs tree of Fig. 2(b), with imposition of a global molecular clock. The tree in panel (b) has been scaled to have the same size on the page for divergence of the Old and New World LCVs as that in panel (a) has for divergence of the Old and New World primates. The tentative timescale (in grey) for panel (b) is transferred from panel (a); it assumes the same date for separation of Old and New World LCV lineages as for separation of Old and New World primate lineages.

these absences, although extinction might be a possible scenario. In this context, it should be noted that the two known types of EBV are closely related strains that both belong in Clade B (McGeoch & Gatherer, 2007).

The evolutionary history of EBV, and the observation that EBV can also be experimentally transmitted to foreign primate hosts (Frank *et al.*, 1976; Cleary *et al.*, 1985), indicates that the species specificity of LCVs is not absolute. Rather, LCV, may be horizontally and zoonotically transmittable. The most recent transmissions evidently occurred about 1 MYA from macaques to hominoids (orang-utans and gibbons) in Indonesia, resulting in the emergence of PpygLCV2 and SsynLCV2. In the wider picture of the mammalian herpesviruses, horizontal transmissions (either observed *in vivo* or

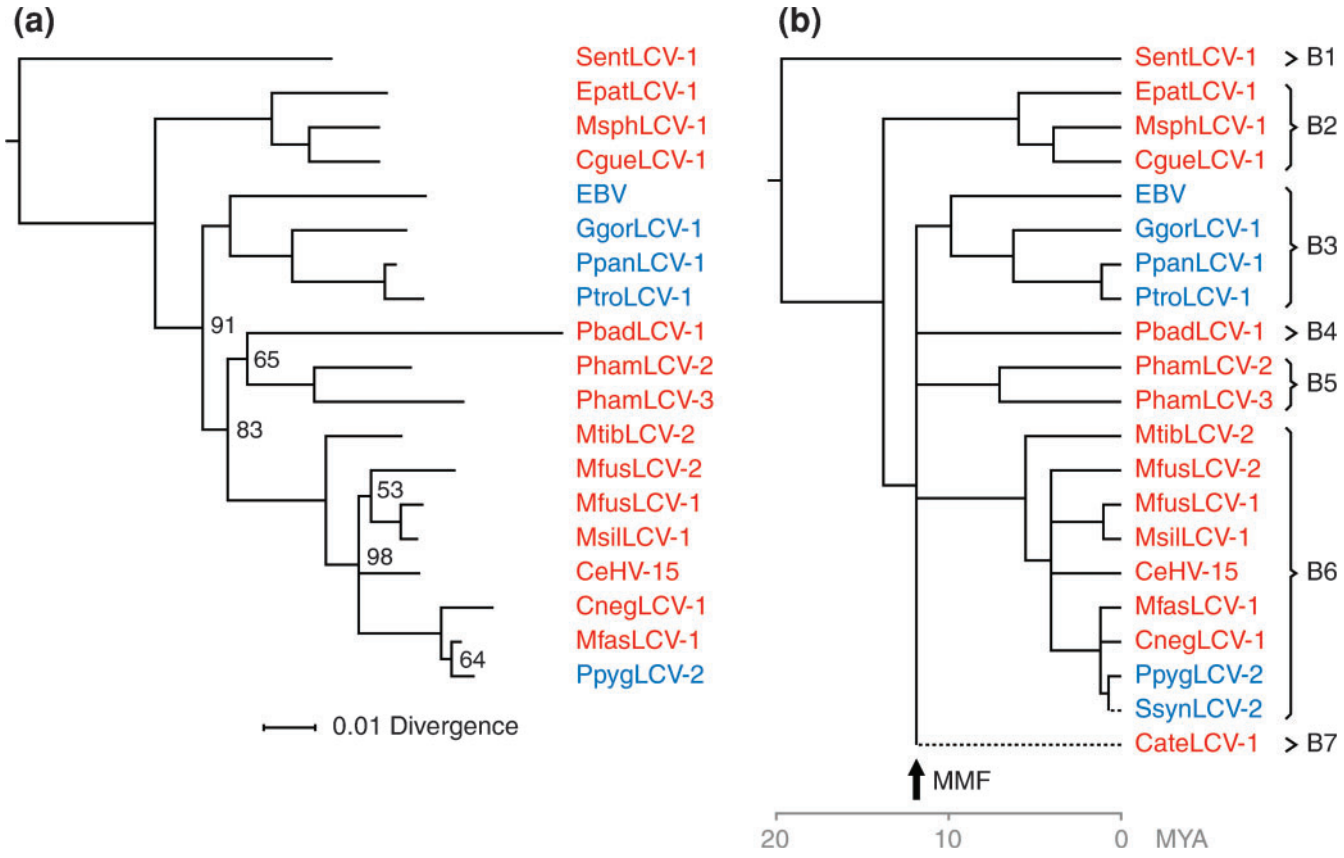

Fig. 4. Phylogenetic tree for Clade B LCVs. (a) Best achievable BMCMC tree for Clade B LCVs. A 1042 residue alignment of concatenated partial gB and DPOL amino acid sequences for 28 LCVs was constructed and analysed by BMCMC. Clades A and C were used to provide a root for Clade B, and are not shown. The Clade B tree is shown as a majority-rule consensus tree, with one multifurcated locus. Resolved nodes had posterior probability of 1.00, except for six which had lower posterior probabilities; the posterior probability (as %) is shown to the right of each of these six nodes. LCV names are coloured as for Figs 2 and 3. A scale indicating divergence, as substitutions per site, is at the foot. (b) Molecular clock tree for Clade B LCVs. The tree shown in panel (a) was reduced to a multifurcated version at all nodes with posterior probability less than 0.91, and a molecular clock version computed (including Clades A and C). Two additional LCVs (CateLCV-1 and SsynLCV-2, for which lesser amounts of data were available) were interpolated into the molecular clock tree on the basis of additional tree-building exercises, and are shown with their terminal branches as dashed lines. The node at the base of the MMF is marked with a heavy arrow. Subclades are annotated as B1–B7. The tentative timescale (in grey) sets the node for divergence of the SentLCV-1 lineage from other Clade B LCVs as corresponding to the divergence of the *S. entellus* lineage from lineages, leading to species of *Macaca*, *Papio*, etc.

deduced from evolutionary studies) are not a rare phenomenon. A well known example is the herpes B virus: in its natural host (macaques) it is benign, but upon transmission to humans it is associated with high mortality (Palmer, 1987). Also, the human herpes simplex virus can be transferred to non-human primates, thereby occasionally killing complete groups of captive individuals (Mätz-Rensing *et al.*, 2003). Pseudorabies virus, which naturally infects pigs, can transmit to dogs, cats and other carnivores with rabies-like symptoms (Mettenleiter, 2008). The gamma-2 herpesviruses alcelaphine herpesvirus 1 and ovine herpesvirus 2 are both asymptomatic in their hosts (wildebeest and sheep, respectively) but cause malignant catarrhal fever (MCF), an often fatal disease, in cattle (Ackermann, 2006). Ovine herpesvirus 2 also causes an MCF-like disease in pigs (Albini *et al.*, 2003). Several other indications for herpesvirus transmission to foreign hosts have been published (Leendertz *et al.*, 2009; Ehlers *et al.*, 2008; Richman *et al.*, 1999; Huang *et al.*, 1978; Meléndez *et al.*, 1969). Taken together, this knowledge indicates a zoonotic potential for herpesviruses, and our present results show that this is also the case for LCVs. Herpesviruses appear not to break the species barrier as often and readily as some RNA viruses. However, as exemplified by the emergence of human immunodeficiency viruses from origins in various simian immunodeficiency viruses (Hahn *et al.*, 2000), in countries with populations of non-human primates, the frequent handling of primates,

their meat and organs might facilitate the zoonotic transmission of herpesviruses from monkeys and apes to humans.

METHODS

Sample collection and processing, PCR methods and sequence analysis. Over 6 years, blood, tissue and faeces samples (n=502) were collected from live or deceased individuals of 49 primate species (apes, Old World monkeys, New World monkeys and prosimians) in the Taï National Park of Côte d'Ivoire (Leendertz *et al.*, 2006), Cameroon, Republic of Congo, Democratic Republic of Congo, Uganda, Indonesia and Vietnam. Samples were also collected from live or deceased individuals in several German zoological gardens and primate facilities. The primate species that yielded herpesvirus sequence data are listed in Table 1, and details of the samples are available on request. DNA was prepared with the QiaAmp tissue kit (Qiagen) according to the manufacturer's instructions.

For universal detection of herpesviruses, pan-herpes DPOL PCR for amplification of 160–181 bp (excluding primer-binding sites) of the DPOL gene was carried out as described previously (Chmielewicz *et al.*, 2003). For detection of the MDBP and gB genes, LCV sequences were amplified with five degenerate, deoxyinosine-substituted primer sets in a nested format (Supplementary Table S1, available in JGV Online). These sets were based on the published CalHV-3 BALF2 gene (Rivailler *et al.*, 2002) (primer set BALF2A) and the PtroLCV-1 gB gene (set BALF4A), or on the PpygLCV-1 BALF2 gene (set BALF2B), the PpygLCV-1 BALF4 gene (set BALF4B) and the GgorLCV-2 BALF4 gene (set BALF4C), as determined in this study. The primer-binding sites were placed in regions conserved among the gammaherpesviruses. The primers were only minimally degenerate in order to avoid amplification of gamma-2 viruses. PCR was carried out at an annealing temperature of 46 °C under conditions used in pan-herpes DPOL PCR. LD-PCR was performed in a nested format with the TaKaRa-Ex PCR system (Takara Bio Inc.) according to the manufacturer's instructions, using virus-specific primers (not listed). PCR products were purified by using the PCR purification kit (Qiagen) and directly sequenced with the Big Dye terminator cycle sequencing kit in a 377 DNA automated sequencer (Applied Biosystems).

Provisional nomenclature, abbreviations and nucleotide sequence accession numbers of novel herpesviruses. Names and abbreviations for newly detected LCVs were formed from the host species name and the genus to which the virus was tentatively assigned (for example: ***P*an *tro*glodytes** **l**ympho**c**ryptov**i**rus, PtroLCV), and are listed with GenBank accession numbers in Table 2. LCVs with published sequences that were used in the analyses and LCVs (Ehlers *et al.*, 2003) from which additional sequences were generated for this study are also listed (Table 2).

Phylogenetic analysis. Amino acid sequence alignments for sets of herpesvirus sequences were made using MAFFT (Katoh *et al.*, 2002). Regions in alignments that were considered too variable to be confidently alignable, plus locations containing a gapping character in any sequence, were removed before using alignments for phylogenetic inference. The amino acid substitution table of Jones *et al.* (1992) was used in tree inference programs.

Phylogenetic trees were inferred from alignments of amino acid sequences. Preliminary trees were derived by the neighbour-joining method using PROTDIST and NEIGHBOR (PHYLIP suite v 3.63; Felsenstein, 1993). Phylogenetic relationships were investigated in depth by compute-intensive Bayesian analysis with BMCMC (MrBayes v 3.1; Ronquist & Huelsenbeck, 2003). Default values for priors were used. For concatenated alignments of gB and DPOL sequences, the two datasets were treated in separate partitions. Substitution rates were modelled as a discrete gamma distribution of four classes plus one invariant class. MrBayes runs were for at least one million generations, and comprised two processes each of one unheated and three heated chains. Trees were sampled every 100 generations and a large burn-in (usually 5001 trees) was applied. Majority rule consensus trees were obtained from the output.

Molecular clock versions of previously derived trees were computed by maximum-likelihood methods with CODEML (PAML suite v 4; Yang, 2007), with substitution rates modelled as a discrete gamma distribution of five classes. Timescales for molecular clock gamma-herpesvirus trees were applied with a single calibration point proposed by reference to the phylogenetic tree of primate host lineages. The comprehensive study of Bininda-Emonds *et al.* (2007) was used as the reference for primate phylogeny and divergence dates. It should be noted that divergence dates from Bininda-Emonds *et al.* (2007) are generally older than those from earlier studies (Schneider, 2000; Raaum *et al.*, 2005; Steiper & Young, 2006), which were used in our recent papers (McGeoch *et al.*, 2006, 2008; Ehlers *et al.*, 2008; Leendertz *et al.*, 2009).

Sequence alignments employed in this work are available, with inferred trees, on request to d.gatherer@mrcvu.gla.ac.uk.

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