Viral forensic genomics reveals the relatedness of classic herpes simplex virus strains KOS, KOS63, and KOS79

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Abstract

Herpes simplex virus 1 (HSV-1) is a widespread global pathogen, of which the strain KOS is one of the most extensively studied. Previous sequence studies revealed that KOS does not cluster with other strains of North American geographic origin, but instead clustered with Asian strains. We sequenced a historical isolate of the original KOS strain, called KOS63, along with a separately isolated strain attributed to the same source individual, termed KOS79. Genomic analyses revealed that KOS63 closely resembled other recently sequenced isolates of KOS and was of Asian origin, but that KOS79 was a genetically unrelated strain that clustered in genetic distance analyses with HSV-1 strains of North American/European origin. These data suggest that the human source of KOS63 and KOS79 could have been infected with two genetically unrelated strains of disparate geographic origins. A PCR RFLP test was developed for rapid identification of these strains.

Keywords: KOS; KOS63; KOS79; HSV-1; genome; variation; genetic distance; co-infection

Introduction

Infection with human herpesvirus 1, or herpes simplex virus 1 (HSV-1; genus *Simplexvirus,* subfamily *Alphaherpesvirinae,* family *Herpesviridae,* order *Herpesvirales*) is widespread in humans, and is the causative agent of recurrent herpes labialis or genitalis (1). Initial infection takes place at epithelial or mucosal surfaces, where virus replicates actively to form self-limited lesions that involve interactions with innate and adaptive immunity (1). HSV-1 also enters sensory nerve endings and migrates in axons of the peripheral nervous system (PNS) by retrograde transport to neuronal nuclei located within sensory or sympathetic ganglia (2–4). It is in this cell population that HSV-1 establishes latency. The virus may spontaneously reactivate and produce intermittent shedding and/or clinical diseases throughout life (3, 5). Occasional invasion of the central nervous system (CNS) by HSV-1 can result in rare but severe and sometimes fatal encephalitis (6, 7). While antiviral drugs have been developed for HSV, these are only able to target the lytic replication stages and the intermittent cycles of productive viral replication (8–11). The latent state is largely refractory to current antivirals (8, 12). HSV remains the most studied herpesvirus, and knowledge of HSV has driven key advances in our fundamental knowledge of the mechanisms of herpesvirus entry, replication, gene expression, assembly and egress (1, 12–14).

The vast majority of laboratory and genetic studies have been restricted to a relatively small number of HSV-1 strains and isolates, of which some of the most common are HSV-1 strains F, KOS, 17, SC16, and McKrae (1, 15–21). Strains of HSV differ greatly in phenotype, particularly in *in vivo* animal model systems of pathogenesis (18, 22–26). Variants of the same name or source are often presumed to be similar, but may show different genetic and phenotypic

characterizations, demonstrating that this assumption does not always hold true (22, 27). For example, strains H166 and H166syn are distinctly different strains, both in phenotype and genotype, but arose from the same source patient (28, 29). Two recently published genome sequences of HSV-1 McKrae, which were based on strains propagated in different labs, revealed coding differences in genes such as UL36 and UL56 (30, 31). We recently demonstrated that clonal variants of strains KOS and F could differ in plaque phenotype and harbor numerous genetic differences despite their overall clonal identity (29). The genomes of five variants of KOS have been published (**Table 1**) (29, 32–34), and many more variants have been utilized in prior studies (15, 22, 34–37), before genome sequencing was a viable option. The history and genome sequences of several of these variants have recently been documented by Colgrove *et al* (34). These KOS variants have been noted to differ in virulence in animal models, as well as other phenotypes, but the genetic basis of these differences is not yet known (22, 29, 34, 38).

Two variants of HSV-1 KOS, called KOS63 and KOS79, were noted relatively early on to differ dramatically in phenotype, both *in vitro* and *in vivo* (22). Limited genetic analyses using restriction fragment length polymorphisms (RFLP) and single-gene analysis by PCR suggested several differences. HSV-1 KOS63, which has an orolabial origin, has been passaged numerous times in multiple laboratories (15, 22, 34, 35, 37, 38), and is believed to represent the standard KOS isolates that circulate in many laboratories, but this has not been clearly established before this work. Another orolabial isolate attributed to the same individual, KOS79, has undergone minimal passage in the laboratory (22). In a matched comparison using both peripheral and intracerebral routes of HSV-1 infection, Dix *et al.* found that KOS79 was highly neurovirulent and neuroinvasive in mice, while KOS63 was much less so (22). KOS63 and KOS79 were

suggested to be distinct strains on the basis of polypeptide synthesis and RFLP analyses, although the primary data were not shown (22). KOS79 has also been shown to harbor an extended array of tandem repeats in the neurovirulence protein ICP34.5, relative to a shorter array found in a plaque-purified variant of KOS called KOS321 (36, 39). A number of differences in the ICP0 promoter region of KOS and KOS79 have also been noted (40).

We set out to address the relatedness of the historical isolate KOS63, and the more virulent strain KOS79. We sequenced the full genomes of KOS79 and KOS63 and compared them to five published genome sequences of KOS (29, 32–34). We found that KOS63 and previously sequenced KOS variants are closely related to one another, akin to clonal variants from a parental population (29). However KOS63 and its clonal variants were all genetically distant from KOS79 – differing as much as unrelated strains isolated from individuals in disparate geographic regions. KOS79 clusters most closely with North American and European strains, while KOS63 and its variants cluster with Asian strains. These data provide further evidence that individuals may harbor quite disparate viral strains, and will enable future evaluations of the genetic basis of the neurovirulence of strain KOS79.

Results

Full genome sequencing of KOS79 and comparison to KOS

We used genome-wide comparative sequence analysis to illuminate the genetic relatedness of HSV-1 KOS79 to HSV-1 KOS63 and other previously described isolates of the KOS strain (**Table 1**). To do this, we first applied Illumina high-throughput sequencing to obtain sequence

read data from viral nucleocapsid DNA of strains KOS79 and KOS63. Consensus genomes were generated for each strain using a previously described combination of *de novo* assembly and reference-guided assembly methods (**Table 2**; see Methods for details) (29). The genomes of KOS79 and KOS63 do not contain any truncated or missing proteins, with the exception of US9 in KOS63. The presence of a SNP (C>T) in US9, which generates an early stop codon that truncates the protein, has been described in multiple prior sequences of the KOS strain (29, 32, 41). Finding this identical SNP in KOS63 supported the premise that it is indeed closely related to the previously sequenced KOS isolates.

To analyze the genetic relatedness of KOS79, KOS63, and the previously sequenced KOS strains from our lab and others, we compared the identity and number of variant proteins among these strains (**Table 3** and **Supplementary Table 1**). As an out-group, we included for comparison the unrelated HSV-1 reference strain 17. We observed that the newly sequenced KOS63 and previously sequenced KOS variants shared an average of 99.2% DNA identity (**Table 3**). In contrast, comparison of KOS63 or the other KOS variants to either KOS79 or strain 17 resulted in a lower DNA identity of 98.5%. This minor shift in percent identity across the genome translated into a sizable number of proteins harboring coding variations in each pairwise comparison. Between KOS63 and the KOS variants, an average of just 6 proteins differ in any pairwise comparison (**Table 3**; see **Supplementary Table 1** for complete list of variations). This is akin to what was previously observed for sister clones from a common parental virus stock (29). In contrast, pairwise comparison of any of these vs. KOS79 or strain 17 yields a similarly high number of over 60 proteins that harbor coding variations in each pairwise comparison (**Table 3**). Nine proteins are completely conserved between KOS79 and any of the KOS63-like

variants: UL15, UL16, UL20, UL26.5, UL33, VP26 (UL35), UL45, VP22 (UL49A), and UL55, several of which were also highly conserved in our prior global analysis of more than 20 independent HSV-1 strains (42). Overall, the amount of difference observed in these comparisons suggested that KOS79 is as different from KOS63-like strains as the unrelated strain 17.

Since percent DNA identity does not reflect the location or distribution of differences between these genomes, we next estimated the evolutionary distance between these strains. As anticipated, the KOS63-like strains had the smallest genetic distance between them in any pairwise comparison, correlating with their high DNA identity (**Figure 1**). Comparison of either KOS79 or strain 17 to any of the KOS63-like variants resulted in a larger estimate of genetic distance, in keeping with the lower DNA identity (**Figure 1**). SplitsTree analysis of KOS79, KOS63-like variants, and strain 17 confirmed the close relationship among the KOS63-like variants, and placed KOS79 and strain 17 at nearly equidistant points away from the KOS63-like variants (**Supplementary Figure 1)**. The distance between KOS79 and any of the other KOS variants suggests that it is a distinct and unrelated strain.

Genetic distance analysis and the likely geographic origin of strain KOS79

In a previous comparison of HSV-1 genomes from diverse geographic areas, we found that a majority of strains clustered near those from the same geographic origin (42). The KOS_{Davido} isolate included in that publication was the sole exception, where despite its USA origin, KOS_{Davido} clustered with strains of Asian origin (42, 43). To place KOS79 within the context of this study, we integrated KOS79 and KOS63 into the prior 26-genome alignment and computed

the genetic distance among these strains. In this analysis, KOS79 clustered with 100% bootstrap confidence with HSV-1 strains F and H129, which were also isolated in the USA (**Figure 2**). This trio is found within a larger 100% bootstrap confidence cluster, which includes three other European and North American strains, HF10, 17 and McKrae. This contrasts with KOS63 and KOS_{Davido}, which are assigned to the Asian genetic distance cluster with equal confidence. Although attributed to the same individual, KOS63 and KOS79 are fundamentally distinct strains that appear to have distant geographic historical origins.

The extensive recombination that is postulated to occur in HSV precludes a cladistic analysis using these data on genetic distance (42, 44–48). To investigate the possibility of recombination between KOS63 and KOS79 in the original host, we performed SimPlot analysis using KOS79, KOS63, a consensus of the European cluster (strains HF10, 17, McKrae, F and H129) and a consensus of the Africa-1 cluster (strains E08, E12, E13, E14 and E19) from our prior study (**Supplementary Figure 2A**) (42). We observed that KOS79 is most closely related to the European consensus over most of its length, except for a few short regions where it is most similar to the Africa-1 consensus (e.g. between kilobases 45-50 and 97-102) or where no predominant background can be identified (e.g. between kilobases 60-65 and 80-85). The corresponding BootScan plot (**Supplementary Figure 2B**) shows chi-shaped crossover signals supportive of recombination with an African strain at 97-102 kb, although the other two areas of African similarity have less well formed signs of crossover. BootScan analysis suggests potential recombination between KOS79 and KOS63 at 60-65 kb and 80-85kb, though this finding should be viewed with caution because the SimPlot analysis implies genetic equidistance between all strains in these regions. Accurate recombination analysis in HSV-1 will always be complicated

by the deep evolutionary history of the species, the extent of recent intercontinental travel with plentiful opportunities for recombination, and the fundamental genetic similarity of all known strains (e.g. more than 98.5% identical over any 10 kb window).

Rapid PCR test to distinguish KOS79 and KOS63

Previous analyses have often grouped KOS63 and KOS79 together (39, 40), although the genomic data now indicate that these viruses are likely to be significantly different. Since the possibility exists that some labs may have initiated studies with a KOS isolate derived from either KOS63 or KOS79, we considered it important to develop an easy assay to distinguish these strains. We therefore designed primers to amplify segments of the tegument proteins UL14 and UL25 (**Supplementary Table 2**), which each contain a strain-specific restriction digest site for *NcoI* or *NdeI* (**Figure 3**). The KOS79 genome contains an *NcoI* site in UL14 while KOS63 and its variants do not (**Figure 3A**). KOS63 and its variants contain an *NdeI* site in UL25 that is not present in KOS79 (**Figure 3C**). To confirm these sequencing results, nucleocapsid DNA from HSV-1 strains 17, KOS63, and KOS79 were used to PCR amplify these regions of UL14 and UL25. These amplicons were then subjected to restriction digest and analyzed. Examination of digestion products revealed the expected patterns (**Figure 3B,D**). Sanger sequencing was used to confirm ten additional SNPs in the UL14 amplicon, and one additional SNP in the UL25 amplicon, that differ between KOS63 and KOS79 (data not shown). This diagnostic tool can be implemented to quickly screen and identify KOS virus stocks or recombinant derivatives within any laboratory as being either KOS63-like or KOS79.

RFLP analysis of KOS strains

To validate the newly assembled genomes for KOS63-like and KOS79 using an alternative method, actual RFLP patterns were compared to those predicted from each genome sequence. Computational comparison of KOS63 and KOS79 predicted the loss of a *HindIII* restriction site in glycoprotein M (gM; UL10) in strain KOS79, resulting in the loss of a 2.8 kb band that exists in KOS63-like variants. In the historical lettering system for HSV-1 DNA restriction fragments, this 2.8 kb band was referred to as the "O" fragment (49–51) (**Figure 4A**). As expected, the 2.8 kb "O" fragment was not present after *HindIII* digestion of KOS79 DNA, but was present in strains KOS and 17 (**Figure 4B-C**). This O-I fusion event has been previously noted to occur in African and European strains of HSV-1, but not in Asian strains (52). Finding the fusion in KOS79, but not KOS63, correlates with the geographic history suggested for each strain by genetic distance analysis (**Figure 2**). Taken together, these results indicate the wide genetic divergence of KOS79 and KOS63, and highlight that genetic comparisons of these strains will reveal many differences regardless of the phenotype under examination (22, 39, 40).

Discussion

KOS is one of the most extensively used strains in HSV-1 research, particularly in the United States. It was isolated from a volunteer in the 1960s (15, 43). In addition to its use in many mechanistic and genetic studies, the KOS strain has also been used to develop vaccine antigens and vectors (53–56). The full genome sequence of HSV KOS (here called KOS_{Davido}) was first published in 2012 (32), and later duplicated with a high degree of fidelity by the Kinchington

group, using a 1981 master stock obtained from Neal DeLuca at the University of Pittsburgh (JQ780693; here called $KOS_{Kinchington}$) (33), and further detailed by Knipe, Coen and colleagues using a master stock provided by Priscilla Schaffer (here called KOS_{Knipe} and KOS1.1) (34). A plaque-purified isolate of KOS (called KOS_{Large}) was sequenced by our own group using a stock obtained from Hendricks, Kinchington and colleagues (29, 57). Previous work by Iwasaki and colleagues (37) highlighted potential immune-evasion differences in the isolates of HSV-1 KOS held by different labs, but the genetic basis of these variants has not been investigated. Here we present the first analysis of overall genetic identity among the five publicly available genome sequences of KOS, and we extend this comparison to include newly sequenced genomes of the historically-dated strains KOS63 and KOS79. These additional strains have distinct levels of virulence in animal models, as previously characterized by Dix *et al* (22), with KOS79 showing significantly higher levels of neurovirulence and neuroinvasiveness than KOS63.

One of the differences between KOS79 and KOS63 that may contribute to the higher virulence of KOS79 *in vivo* is its intact US9 protein. Previous authors have shown that US9 is involved in anterograde transport of viral capsids and glycoproteins to axonal termini in related alphaherpesviruses, such as pseudorabies virus (PRV) (58–60). However the role of US9 in anterograde transport of HSV-1 is less clear (61–64). KOS63 and its variants have a mutation present in the TATA-box promoter region of the membrane protein US9, as well as a substitution at position 58 in the US9 gene. This substitution introduces an early stop codon, and truncates the last 32 residues of the US9 protein (32, 41). This mutation removes the stop codon and elongates the neighboring open reading frame of US8A, a protein of undetermined function (32, 41). The new availability of complete coding sequences for KOS79 and KOS63 will enable

future studies on how the differences in other herpesvirus proteins contribute to their observed differences in virulence phenotypes.

KOS63 and KOS79 were reportedly isolated from the same volunteer on separate occasions (15, 22, 43). The historical record does not allow us to confirm these isolations or to collect a fresh isolate from the same individual, but the frequent usage of KOS63-like variants in labs around the world makes it imperative to clarify their history as much as possible. We present here a rapid PCR and restriction digest test to allow labs to easily distinguish between KOS79 and the classic KOS63-like strains. In past studies, authors did not distinguish whether KOS63 and KOS79 were passage variants—i.e. high- and low-passage variants from a common parental stock—or were unrelated isolates (40, 39). Here we showed that KOS79 is genetically distinct from KOS63-like strains, with sufficient divergence that one could posit independent geographic acquisitions of HSV-1 infection (43). These data remove the possibility that KOS63 represents a passage variant of KOS79, a hypothesis that arose from examples of attenuation by serial passage for related herpesviruses (65–67). If they arose in the same source, the historical dating of strains KOS63 and KOS79 (from 1963 and 1979 respectively; (22)) suggests that these viruses reactivated independently of one another and at different times. It is impossible to determine now whether these historical isolates also arose from different body sites, which emphasizes the importance of recording full clinical details for new isolates. In future studies of extant cases, it will be important to determine how viral reactivation and shedding occur in dual- or multiply-infected individuals, with attention to aspects of space, time, and genetic variation.

Methods

Virus culture and DNA isolation

Stocks of HSV-1 KOS63 and KOS79 were obtained from R. Dix and expanded in adherent MRC-5 (ATCC®, CCL-171) human fetal lung fibroblast cells. MRC-5 cultures grown in Eagle's Minimum Essential Media (EMEM; Sigma-Aldrich) were infected at an MOI of 0.01 and harvested when cultures displayed significant cytopathic-effect (CPE). These master stocks were titered on Vero cells (ATCC®, CCL-81) for easier plaque visualization. For DNA preparation, MRC-5 cultures were infected at an MOI of 5 and viral genomic DNA (gDNA) was isolated via a nucleocapsid preparation assay previously described (68). Similarly prepared gDNA from KOS_{Large} (29) and from plaque-purified HSV-1 strain 17 were used in the RFLP and PCR studies below.

Next generation sequencing

Viral gDNA was sheared and prepared for sequencing on an Illumina MiSeq. Viral nucleocapsid DNA was sheared using a Covaris M220 sonicator/disruptor, with the following parameters: 60 seconds duration, peak power 50, 10% duty cycle, at 4°C. The Illumina TruSeq DNA sample prep kit was used to prepare barcoded sequencing libraries, according to the manufacturer's low-throughput protocol. Libraries were quantified and assessed by Qubit (Invitrogen, CA), Bioanalyzer (Agilent), and qPCR for library adaptors (KAPA Biosystems). Paired-end sequencing (2 x 300 bp length) was carried out on our lab's Illumina MiSeq instrument, according to manufacturer's recommendations, with 17 pM input. All sequence data has been deposited at the NCBI Short Read Archive under BioProject ID PRJNA295931. Sequence

alignments are also openly available from the Lancaster University data archive at http://dx.doi.org/10.17635/lancaster/researchdata/29.

Consensus viral genomes were assembled using a recently described viral genome assembly (VirGA) workflow (29). Briefly, this workflow performs a number of quality control filters, including removal of contaminating host sequences, adaptors from library preparation, or image-acquisition artifacts. It then carries out multiple iterations of *de novo* assembly using SSAKE, which are combined into longer blocks of sequence (contigs) using Celera and GapFiller. The Mugsy alignment package is used to match these contigs to a reference genome for HSV-1 (strain 17, JN555585). The best matching contigs are stitched into a single consensus genome using a VirGA-specific script called mafnet. Finally, an array of quality-control measures are used to annotate, query, and improve the draft consensus genome. These include comparing the pileup of raw sequence reads to the initial draft genome, detecting polymorphisms within the sequence, identification of sequence features, gaps, and low coverage areas, and validation of selected areas by PCR and Sanger sequencing. Consensus genome sequences for KOS79 and KOS63 have been deposited in GenBank under accessions KT425109 and KT425110.

Pairwise identity and genetic distance comparisons

Trimmed genome sequences, lacking the terminal copies of the large repeats, were used for genetic distance comparisons to avoid giving undue weight to the repeats. In strains KOS_{Davido}, KOS_{Knipe}, and KOS1.1, the lengths of several reiterations (also known as variable number tandem repeats, or VNTRs) have been artificially set to match the reiteration copy number from the reference strain 17 (32, 34). We used the annotation of these VNTRs in the GenBank

accession of KOS_{Davido} (JQ673480) to locate and remove these regions from all pairwise calculations of distance and percent identity. ClustalW2 (69) was used to construct pairwise global nucleotide alignments between whole genome sequences and pairwise global amino acid alignments between ORFs. These alignments were utilized by downstream custom Python scripts to calculate percent identity, protein differences, and genetic distance between samples. Further, an all-vs.-all alignment was used to generate a NeighborNet phylogenetic network in SplitsTree (44, 45, 70) with Uncorrected P distances in order to illustrate potential evolutionary relationships between strains.

Global genetic distance and recombination analyses

KOS79 was incorporated into the genome-wide alignment from Szpara et al. (42) using MAFFT with input parameters specified to freeze the prior alignment (71). Addition of KOS79 and KOS63 did not alter the published alignment of any of the other 26 HSV-1 genomes (42). The source alignment is found on the accompanying data website to Szpara *et al.* (42): http://szparalab.psu.edu/hsv-diversity/. Genetic distances were calculated in MEGA6 (72) using the Maximum Composite Likelihood measure and a dendrogram was produced using the Unweighted Paired Group Mean (Arithmetic) method (UPGMA) with 1000 bootstrap replicates (73, 74). Recombination was analyzed using SimPlot (75).

RFLP analysis

Nucleocapsid DNA was digested overnight at 37^{o}C using the restriction enzymes *BamHI, EcoRI,* and *HindIII* (New England BioLabs), according to the company's specifications. 250 ng of DNA was used in each reaction along with the recommended buffer for each restriction enzyme.

Samples were visualized on a 1.0% agarose gel with ethidium bromide (Fisher Bio-Reagents) after overnight separation. Digital digests of the corresponding draft genomes were carried out using the software Geneious (version 7.1.7, created by Biomatters, available from http://www.geneious.com). These predicted patterns were compared against actual banding from above digestions, to assess draft genome matching to the observed RFLP banding patterns.

Restriction digest of PCR amplicons

Primers were designed flanking *NcoI* and *NdeI* restriction endonuclease cut sites in UL14 and UL25 respectively (Table 1). HSV 17, KOS79, KOS_{Lg} and KOS gDNA was boiled for five minutes and then snap-cooled on ice for five minutes. PCR was conducted in 50 µl reaction volumes using 3 µl of template DNA, 1 unit of PrimeStar GXL DNA polymerase (Clontech), 200 mM deoxynucleoside triphosphate, 1 µM primers, and 1X buffer. PCR conditions in an Eppendorf Mastercycler Nexus gradient were as follows: 98°C for 2 minutes, followed by 35 cycles of denaturation at 98°C for 10 seconds, 15 seconds of annealing at 55°C for UL25 or 60°C for UL14, and primer extension at 68°C for 1 minute. Final extension was carried out at 68°C for 2 minutes. PCR amplicons were then subjected to restriction digest using *NcoI* and *NdeI* (New England Biosciences). 20 µl of PCR mixture containing the amplicon of interest was mixed with 5 µl of enzyme-appropriate buffer, 24 µl of water, and 1 µl of restriction endonuclease. Samples were incubated at 37°C for 1 hour, then heat inactivated at either 65°C for *NdeI* or 80°C for *NcoI*. Digested samples were then purified using a QIAquick PCR purification kit (Qiagen). Standard agarose gel electrophoresis was used to separate and visualize bands. Gels were run at 100 volts for 1 hour at room temperature.

Acknowledgements

We dedicate this work to Dr. Kendall O. Smith, whose early contributions and mentoring of herpes virologists have led to many subsequent discoveries. This work was supported by grants to MLS from NIH-NIAID K22 AI095384 and the Virus Pathogens Resource (ViPR) Bioinformatics Resource Center. PRK was supported by NIH EY015291, EY08098 the Eye & Ear Institute of Pittsburgh and Research to Prevent Blindness Inc. The authors thank L.W. Enquist, in whose lab this work was initiated, and members of the Enquist lab for their feedback during early phases of this work.

Tables

Table 1: KOS variants and strains used for genome sequence comparisons.

Abbreviated name	Parent strain	Accession	Source lab (reference)
KOS_{Davido}	KOS	JQ673480	Davido and colleagues (32)
KOS_{Large}	KOS	KM222721	Szpara and colleagues (29)
$KOS_{Kinchington}$	KOS	JQ780693	Kinchington and colleagues[1]
KOS_{Knipe}	KOS	KT899744.1	Knipe and colleagues (34)
$KOS_{1.1}$	KOS	KT887225.1	Knipe and colleagues (34)
KOS63	KOS	TBD	Dix (22), Szpara and colleagues[2]
KOS79	KOS79	TBD	Dix (22), Szpara and colleagues[2]
17	17	JN555585	McGeoch, Davison, and colleagues (20, 21, 76)

[1]$KOS_{Kinchington}$ is described only by GenBank record; [2]KOS63 and KOS79 are presented for the first time here.

Table 2. Sequencing statistics for HSV-1 strains KOS79 and KOS63.

HSV-1 strain	Paired-end read length	Raw reads	Used for assembly	Genome length	Depth. ≥100	GenBank Accession
KOS79	300 bp	7,761,149	6,079,163	150,782	99.9%	KT425109
KOS63	300 bp	6,293,716	3,483,600	152,389	96.8%	KT425110

Table 3: Pairwise comparisons of genome-wide DNA identity (upper right) and number of proteins with coding variations (lower left) in HSV-1 strains.

# of proteins with coding variations*	Percent pair-wise DNA identity →								
	Strains	**17**	**KOS79**	**KOS63**	**KOS_{Davido}**	**KOS_{Large}**	**KOS Kinchington**	**KOS_{Knipe}**	**KOS1.1**
	17		98.3%	98.4%	99.0%	98.3%	98.5%	98.9%	98.7%
	KOS79	57		98.4%	98.1%	98.6%	98.4%	98.1%	98.1%
	KOS63	61	65		99.2%	99.3%	99.2%	99.1%	99.1%
	KOS_{Davido}	61	65	4		99.0%	99.3%	99.6%	99.7%
	KOS_{Large}	63	65	7	10		99.6%	99.1%	99.0%
	$KOS_{Kinchington}$	61	65	3	2	9		99.3%	99.2%
	KOS_{Knipe}	61	65	5	4	11	3		98.9%
	KOS1.1	61	65	2	5	8	4	6	

* See Supplementary Table 2 for a list of specific proteins that vary between each pair of strains.

Figure Legends

Figure 1: Genome sequence comparisons reveal that KOS63 is as different from KOS79 as from the HSV-1 reference strain 17. The genome sequences of multiple variants of KOS63 (green) were compared to those of KOS79 (blue) and the HSV-1 reference strain 17 (red). For every possible pairwise combination of genomes, we calculated the genetic distance and the percent DNA identity (see Methods for details; see also Table 3). Points are color-coded to reflect the pairs in each comparison, with strain labels next to each point (see below for abbreviations). The x-axis reflects decreasing percent identity, while the y-axis reflects increasing genetic distance. In both cases, more disparate pairwise comparisons will plot further from the origin. Shaded circles provide a visual guide to the groups of pairwise comparisons. Green shading indicates pairwise combinations of KOS63-like strains, blue shading indicates combinations involving strain KOS79, and red shading indicates combinations involving strain 17. Yellow shading indicates the sole pairwise combination of strains 17 and KOS79. Trimmed genome sequences, lacking the terminal copies of the large repeats, were used for all comparisons to avoid giving undue weight to the repeats. Reiteration lengths in several published strains (32, 34) are artificially matched to strain 17, so these regions were removed from all comparisons as well (see Methods for details). Abbreviations are as follows: Da, KOS_{Davido} from Davido and colleagues (32); Lg, KOS_{Large} from Szpara and colleagues (29); Ki, $KOS_{Kinchington}$ from Kinchington and colleagues (33); Kn, KOS_{Knipe} and 1.1, KOS1.1, from Knipe and colleagues (34); 63, KOS63 as described by Dix and colleagues

(22), with genome sequenced here; 79, KOS79 as described by Dix and colleagues (22), with genome sequenced here; 17, HSV-1 reference strain 17, as initially published by McGeoch and colleagues (20, 21), and revised by Davison and colleagues (76). All GenBank accessions are listed in Table 1.

Figure 2: Genetic distance reveals that KOS63 and KOS_{Davido} cluster separately from KOS79 in a global collection of HSV-1 strains.

We extended a prior dendrogram analysis of 26 HSV-1 strains of global distribution (42) to include strains KOS63 and KOS79. KOS63 and the originally-included KOS_{Davido} strain both cluster with HSV-1 strains of Asian origin, while KOS79 groups with HSV-1 strains of European and North American origin. Dendrogram was calculated with UPGMA in MEGA (1,000 bootstrap replicates). Branch confidence values are indicated on the tree. Scale bar indicates the number of nucleotide substitutions per kilobase.

Figure 3: Restriction digest patterns of HSV-1 UL14 and UL25 amplified regions.

Schematic representation of UL14 **(A)** and UL25 **(C)** amplicons including predicted size and location of strain-specific *NcoI* **(A)** or *NdeI* **(C)** sites within each PCR amplicon. The sequence surrounding the distinctive *NcoI* cut site in KOS79 **(A)** and *NdeI* cut site in KOS63 **(C)** are highlighted in yellow, with other HSV-1 strains shown for comparison. Electrophoretic analyses of UL14 **(B)** and UL25 **(D)** amplicons with and without enzyme addition are shown for HSV-1 strains 17, KOS_{Lg}, KOS79, and KOS63. Bands at 0.5kb and 1kb are marked on the 100 bp ladder in each gel.

Figure 4. RFLP analysis of HSV-1 KOS variants.

Diagram of the HSV-1 genome (top) and predicted *HindIII* restriction sites (bottom). The location **(A)** and sizes **(B)** of the historical lettering systems for *HindIII* fragments (49–51) predicted for these genomes are also illustrated. Fusion of fragment O and I, due to loss of a *HindIII* site in gM (UL10), has been previously noted in a number of HSV-1 strains. This loss occurs in strain KOS79, both in the genome sequence **(B)** and in RFLP analysis **(C)** after *HindIII* digestion of viral nucleocapsid DNA. Abbreviations in the genome diagram are as follows: TRL/IRL, terminal/internal repeat of the long region; IRS/TRS, internal/terminal repeat of the short region.

Figures

Figure 1: Genome sequence comparisons reveal that KOS63 is as different from KOS79 as from the HSV-1 reference strain 17.

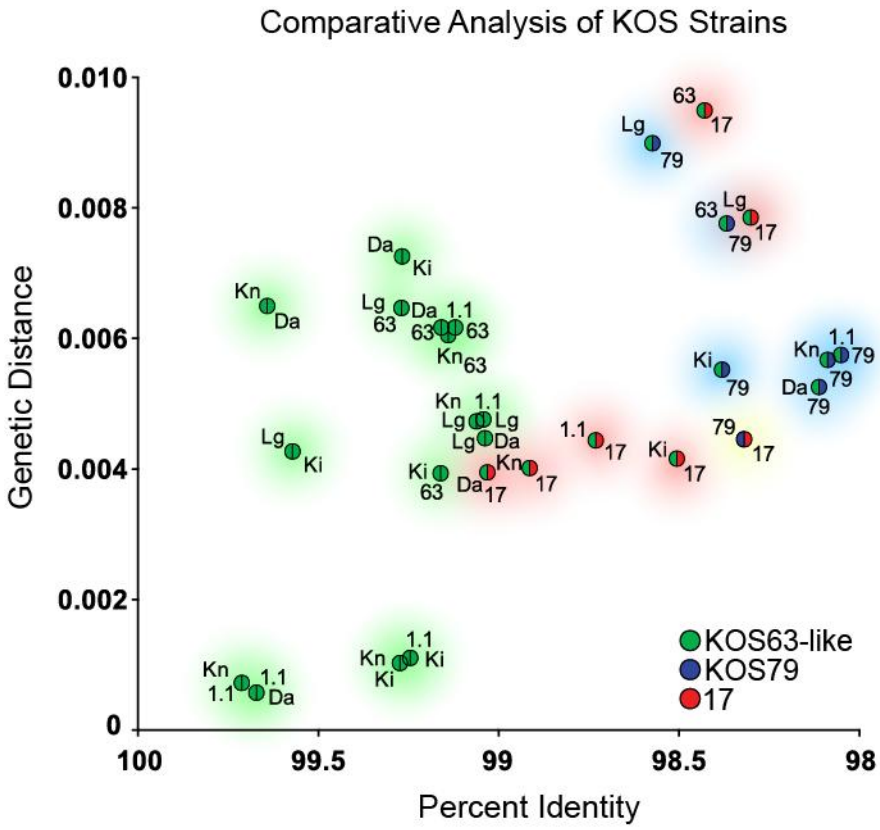


Figure 2: Genetic distance reveals that KOS63 and KOS_{Davido} cluster separately from KOS79 in a global collection of HSV-1 strains.

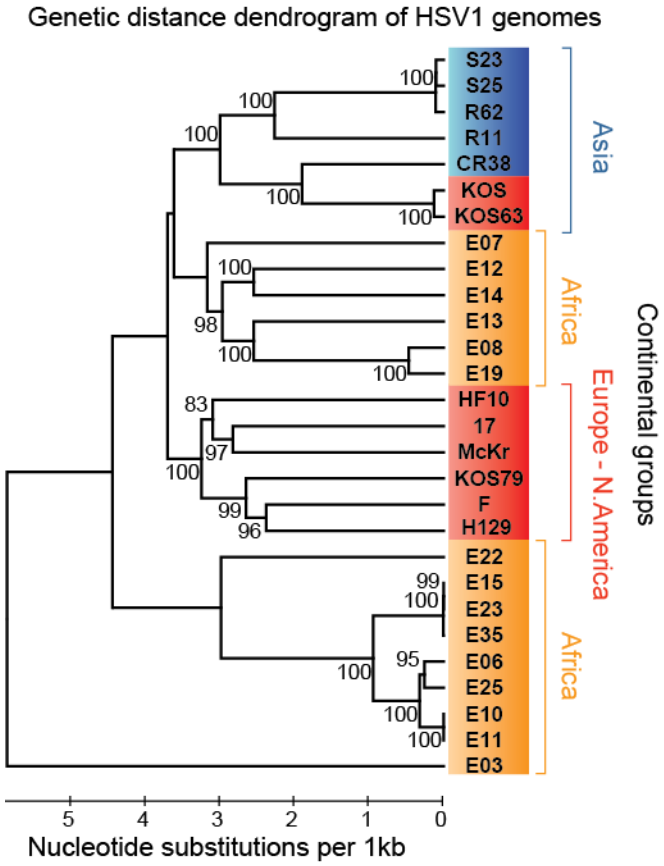


Figure 3: Restriction digest patterns of HSV-1 UL14 and UL25 amplified regions.

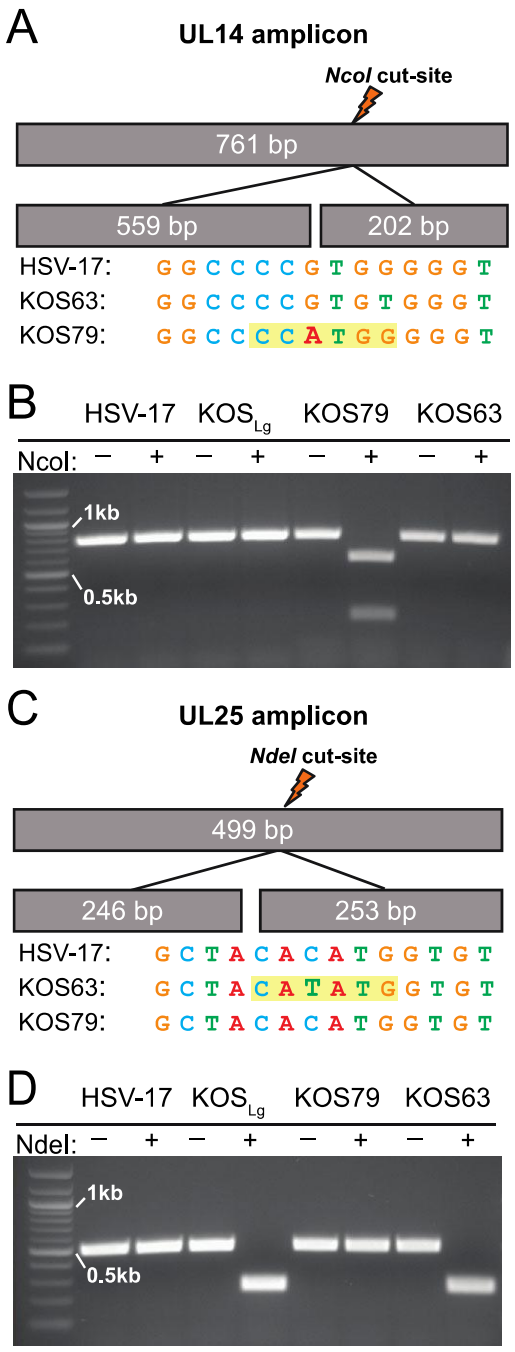
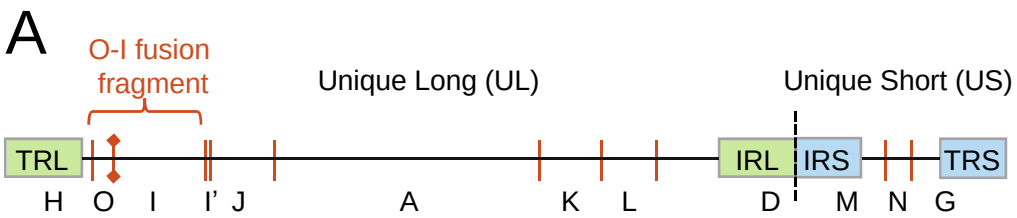
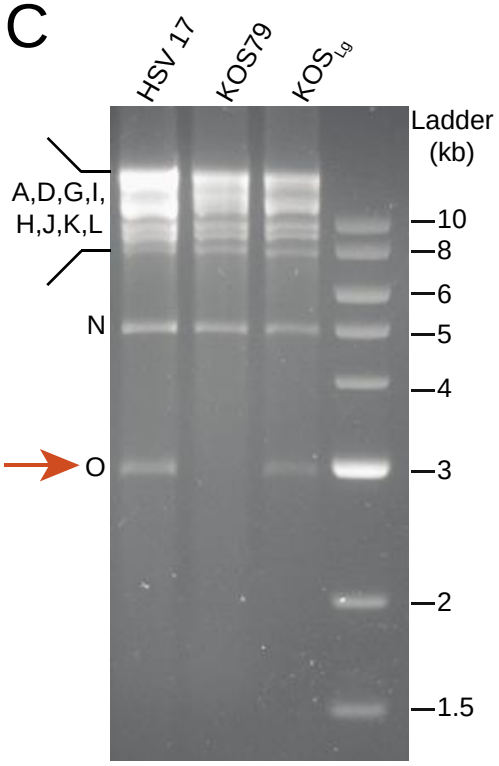


Figure 4. RFLP analysis of HSV-1 KOS variants.



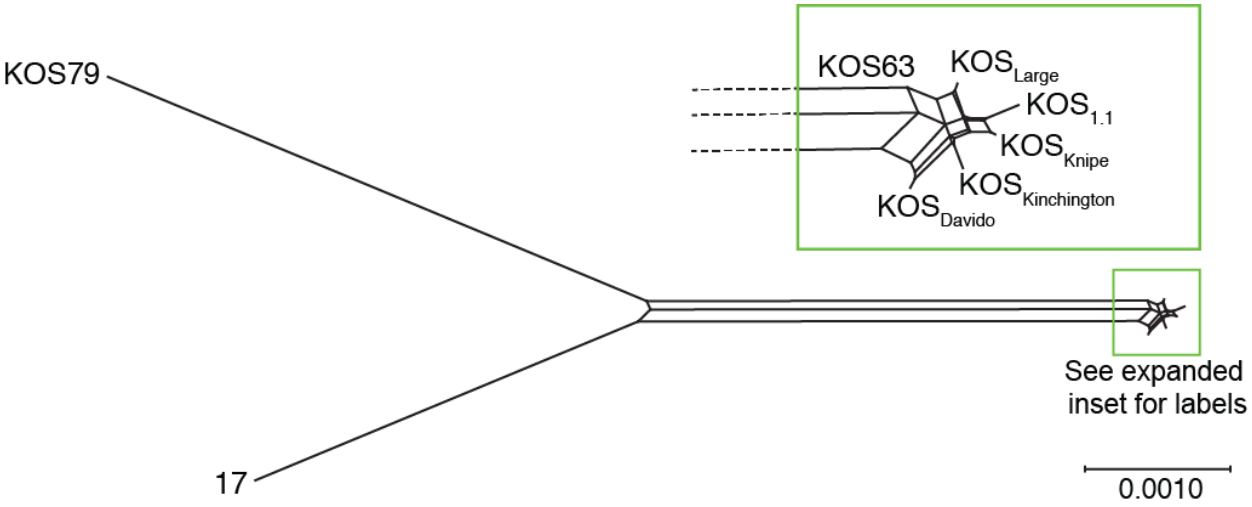
B

HindIII Fragment	HSV-1 17	KOS79	KOS_{Lg}
A	40.8	40.7	40.7
D	34.6	34	33.8
G	13.8	15.5	13.1
I	12.3	15.2	12.3
H	12.7	12.4	12.3
J	11.8	→	→
K	9.4	→	→
L	8.6	→	→
N	4.8	→	→
O	2.8	N/A	2.8
I'	52 (bp)	→	→



Supplementary Figures & Legends

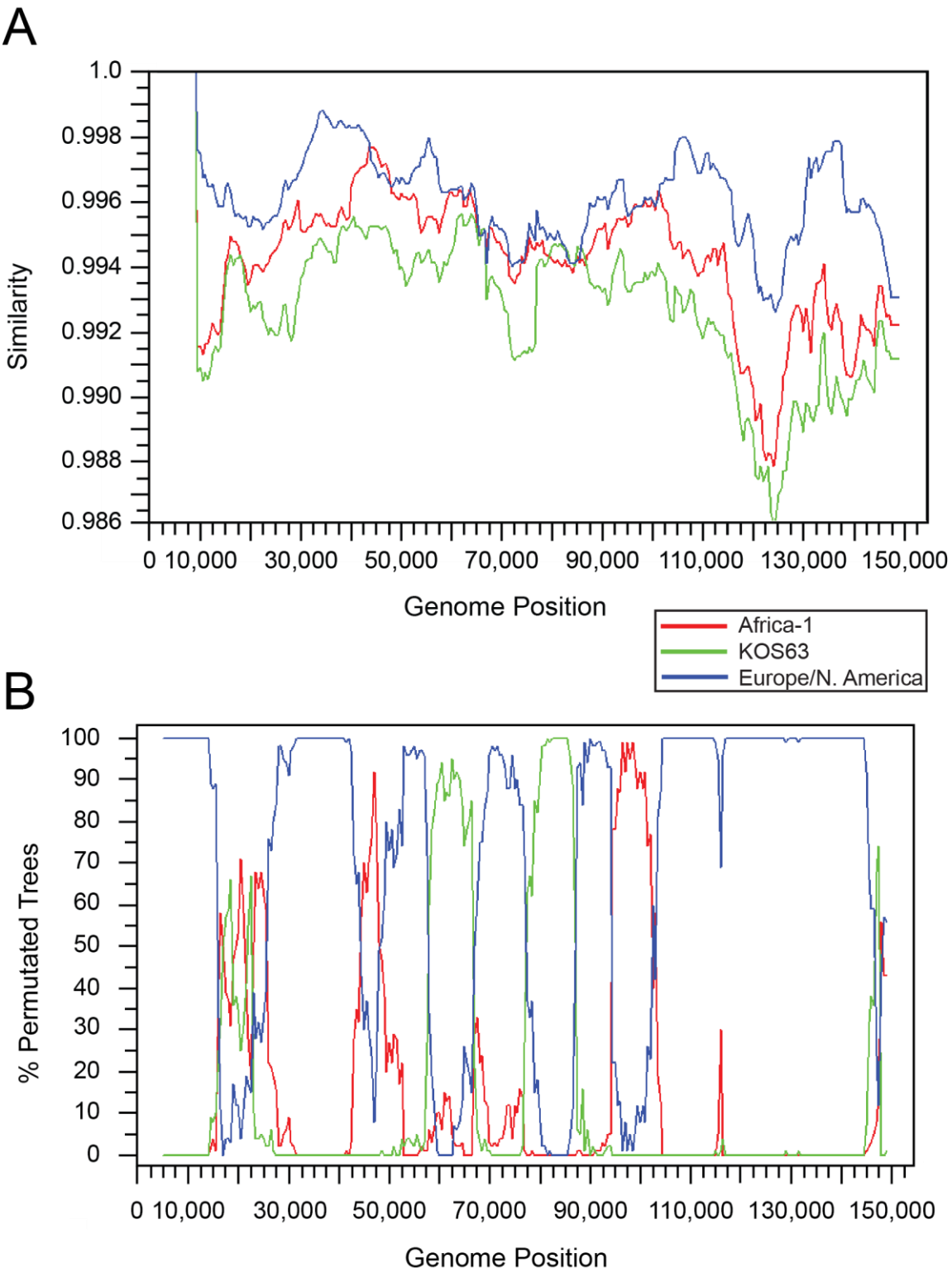
Supplementary Figure 1: SplitsTree analysis of KOS79 and KOS63-like variants.



Supplementary Figure 1: SplitsTree analysis of KOS79 and KOS63-like variants.

SplitsTree analysis (44, 45, 70) reveals the close relationship among the KOS63-like variants, as compared to either KOS79 or the HSV-1 reference strain 17. Inset (green box) provides an expanded view and labels for the compact section of the network containing KOS63-like strains. Trimmed genome sequences lacking the terminal copies of the large repeats that flank each unique region were excluded, so that these genome regions did not doubly influence the genetic relatedness. Likewise, reiterations set to match those of strain 17 were excluded, so that these did not unduly influence the network (see Methods for details). Genome accession numbers and sources are listed in Table 1.

Supplementary Figure 2: Simplot and BootScan comparison of KOS79 with consensus of the European/North American and Africa-1 clusters.



Supplementary Figure 2: Simplot and BootScan comparison of KOS79 with consensus of the European/North American and Africa-1 clusters.

SimPlot analysis **(A)** of KOS79, in comparison to KOS63, a consensus of the European/North American cluster (strains HF10, 17, McKrae, F, and H129), and a consensus of the Africa-1 cluster (strains E08, E12, E13, E14, and E19) from our prior study (Figure 2) (42). KOS79 is most closely related to the European/North American consensus strain over most of its length, except for a few short regions where it is most similar to the Africa-1 consensus (e.g. between kilobases 45-50 and 97-102) or where no predominant background can be identified (e.g. between kilobases 60-65 and 80-85). These regions may represent recombination events, although whether recent or ancient cannot be known. The corresponding BootScan plot **(B)** shows chi-shaped crossover signals supportive of recombination with an African strain at ~97-102 kb, although two other areas of African similarity have less well formed signs of crossover. Potential crossover events with KOS63 occur at 60-60 kb and 80-85 kb **(B)**, although the SimPlot signals in these areas are not strong **(A)**.

Supplementary Tables

Supplementary Table 1: Proteins varying between each pairwise combination of KOS63-like strains.

Strains	KOS63	KOS_{Davido}	KOS_{Large}	KOS Kinchington	KOS_{Knipe}
KOS_{Davido}	gB (UL27), VP1/2 (UL36), RR1 (UL39), ICP4 (RS1)	-	-	-	-
KOS_{Large}	UL8, UL9, UL13, UL21, gH (UL22), VP1/2 (UL36), US6	UL8, UL9, UL13, UL21, gH (UL22), gB (UL27), VP1/2 (UL36), RR1 (UL39), ICP4 (RS1), gD (US6)	-	-	-
KOS Kinchington	gB (UL27), VP1/2 (UL36), RR1 (UL39)	VP1/2 (UL36), ICP4 (RS1)	UL8, UL9, UL13, UL21, gH (UL22), gB (UL27), VP1/2 (UL36), RR1 (UL39), gD (US6)	-	-
KOS_{Knipe}	gB (UL27), VP1/2 (UL36), RR1 (UL39), ICP34.5 (RL1), ICP0 (RL2)	ICP4 (RS1), VP1/2 (UL36), ICP34.5 (RL1), ICP0 (RL2)	UL8, UL9, UL13, UL21, gH (UL22), gB (UL27), VP1/2 (UL36), RR1 (UL39), ICP34.5 (RL1), ICP0 (RL2), gD (US6)	VP1/2 (UL36), ICP34.5 (RL1), ICP0 (RL2)	-
KOS1.1	UL30, VP1/2 (UL36)	gB (UL27), UL30, VP1/2 (UL36), RR1 (UL39), ICP4 (RS1)	UL8, UL9, UL13, UL21, gH (UL22), UL30, VP1/2 (UL36), gD (US6)	gB (UL27), UL30, VP1/2 (UL36), RR1 (UL39)	gB (UL27), UL30, VP1/2 (UL36), RR1 (UL39), ICP34.5 (RL1), ICP0 (RL2)

Supplementary Table 2: Primers for PCR Validation

Primer Name	Target	Sequence
KOS_UL14_F1	UL14	5'-GGGGAACACTATCTGTCGTTGTTGCAGC-3'
KOS_UL14_R1	UL14	5'-GATCGTCTTGCGGACCAGGAGGAGCAA-3'
KOS_UL14_F2_Seq	UL14	5’-GCGTGAGGGTAAGGATGTG-3’
KOS_UL14_F2.1_Seq	UL14	5’-GCGTGAGGGTGAGGATGTG-3’
KOS_UL14_R2_Seq	UL14	5’-CTGGTCATGTGGCAGCTAA-3’
KOS_UL25_F1	UL25	5'-GTCGATATCGAGCGCCGGTTAC-3'
KOS_UL25_R1	UL25	5'-GGTACAGCAGGTAGAGACACAACAC-3'
KOS_UL25_F2_Seq	UL25	5’-CGGAACGTGCACGAGAT-3’
KOS_UL25_R2_Seq	UL25	5’-CGTCATGAAGGTCTTGGACA-3’

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Figure 1

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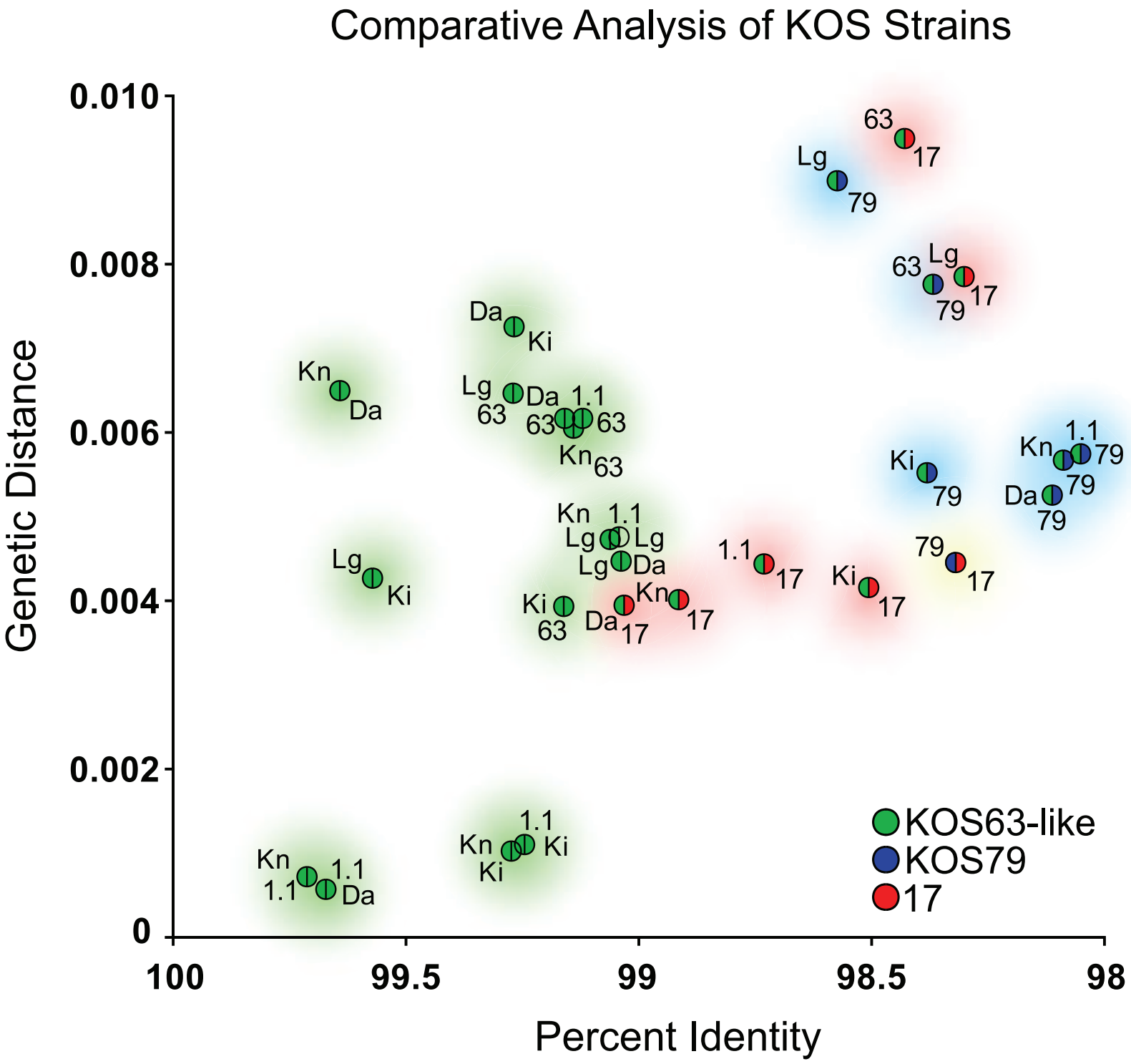

Figure 2

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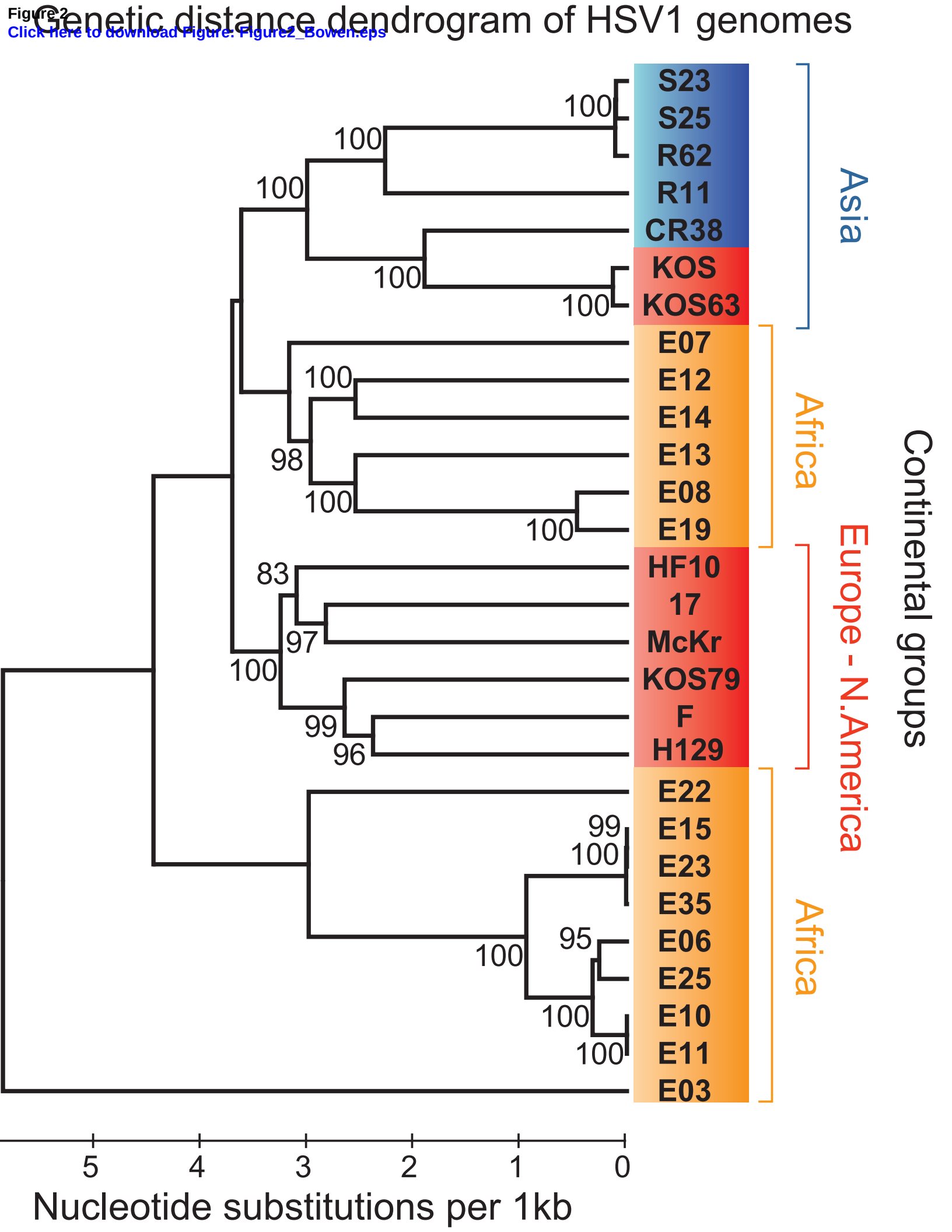

Figure 3
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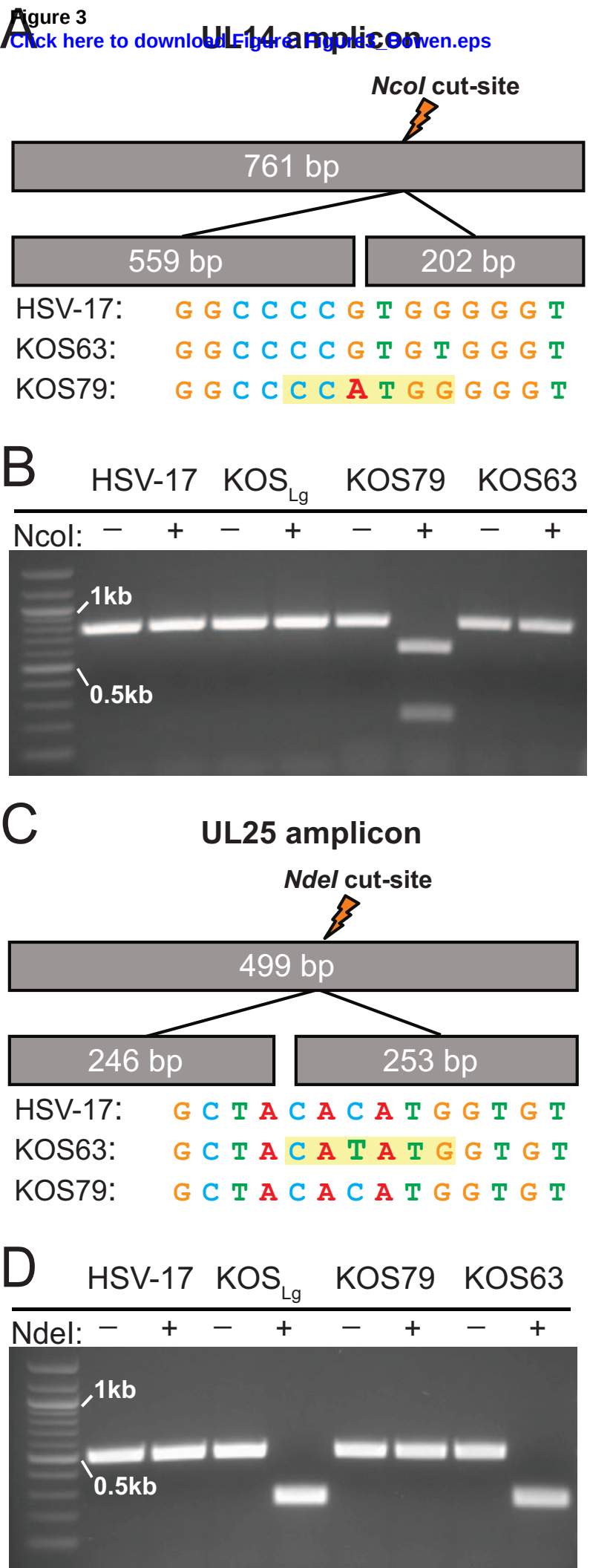
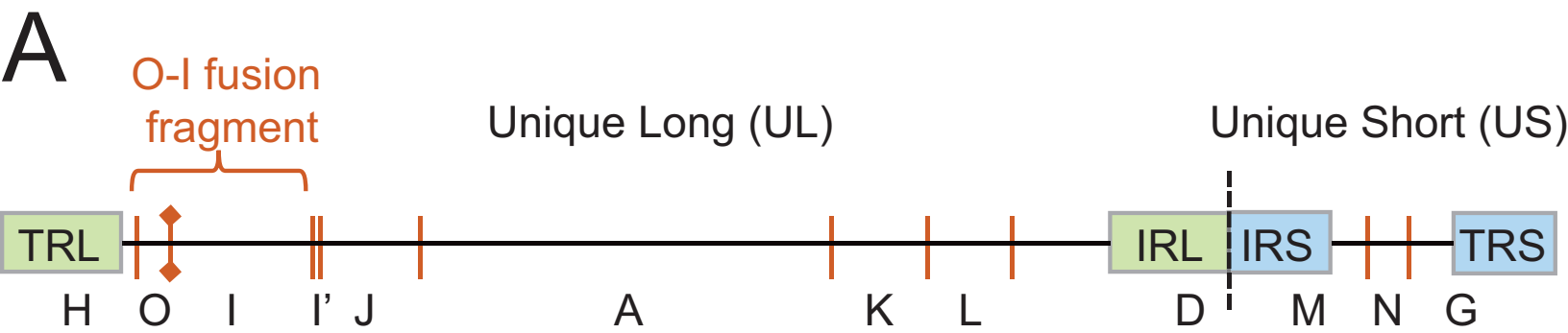


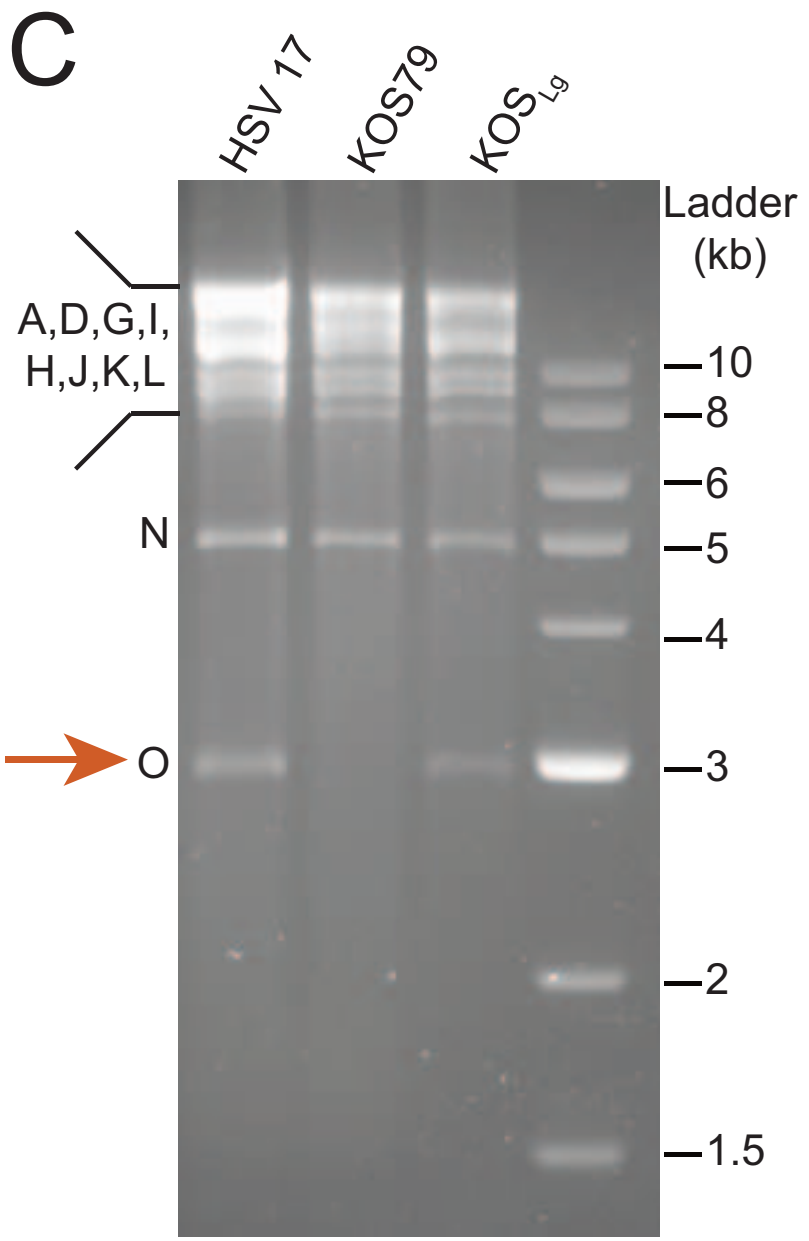
Figure 4

Click here to download Figure: Figure4_Bowen.eps

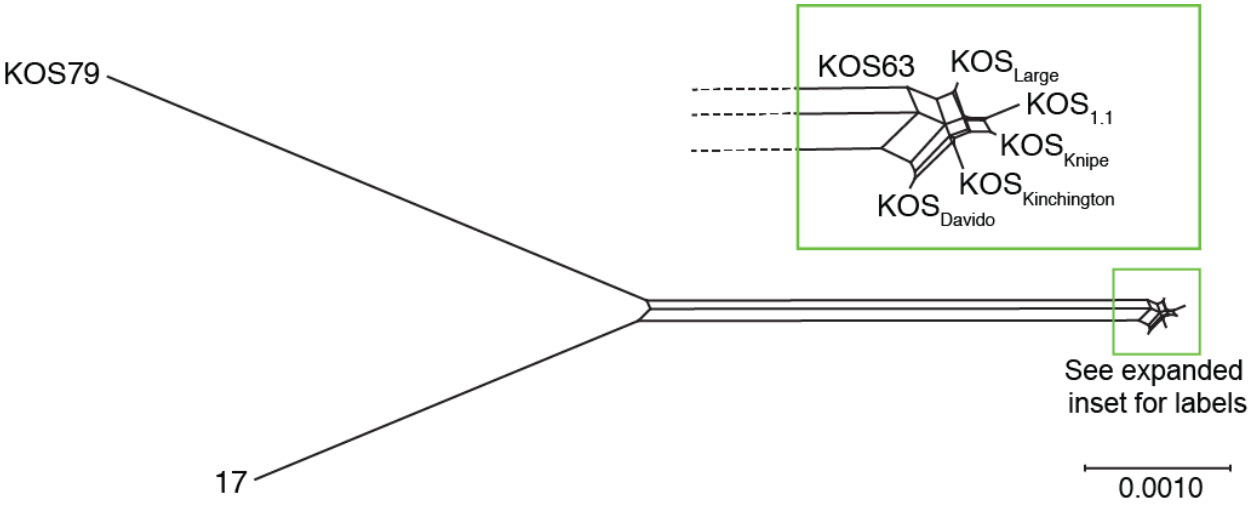


B

HindIII Fragment	HSV-1 17	KOS79	KOS_{Lg}
A	40.8	40.7	40.7
D	34.6	34	33.8
G	13.8	15.5	13.1
I	12.3	15.2	12.3
H	12.7	12.4	12.3
J	11.8	→	→
K	9.4	→	→
L	8.6	→	→
N	4.8	→	→
O	2.8	N/A	2.8
I'	52 (bp)	→	→



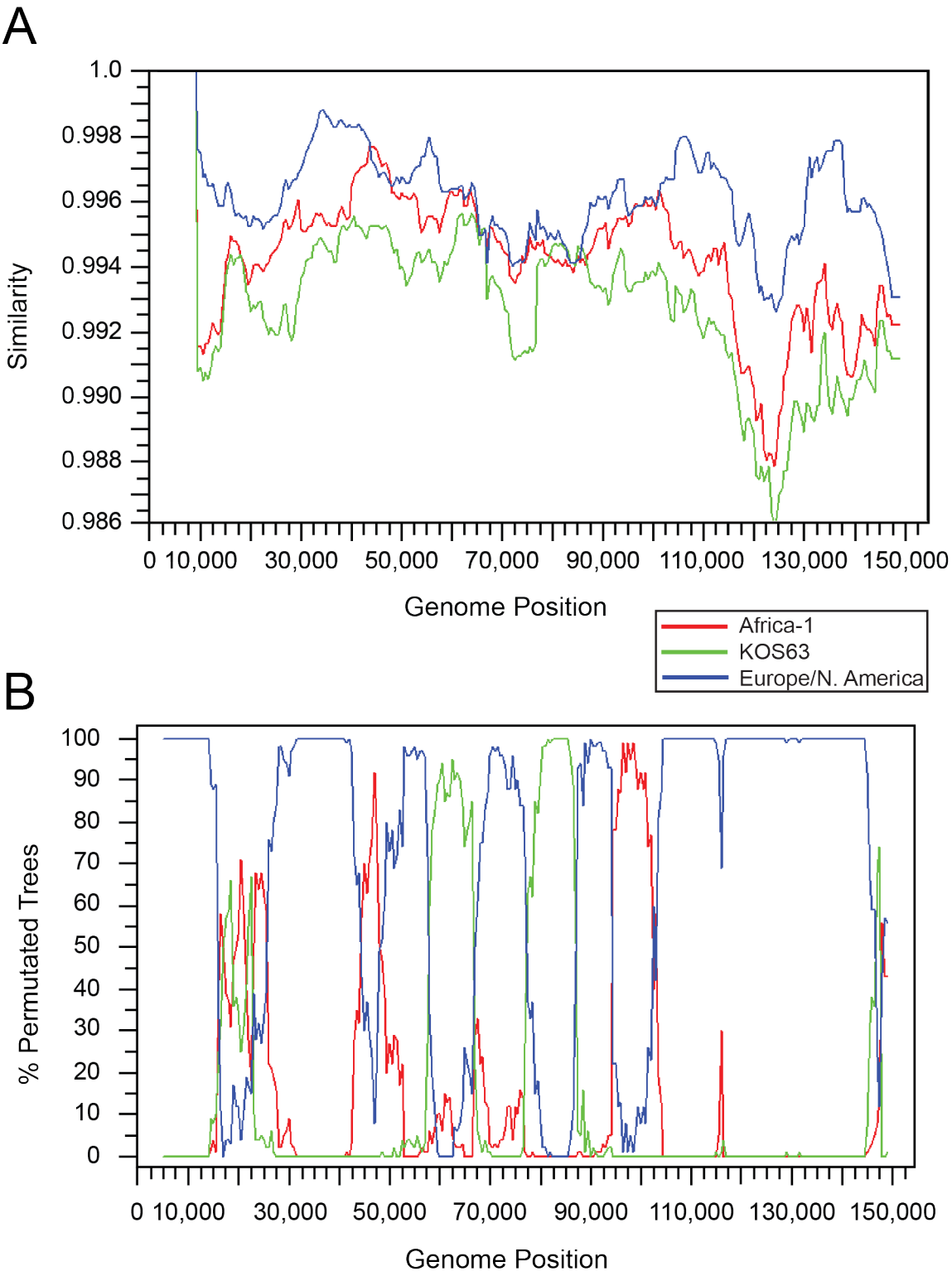
Supplementary Figure 1: SplitsTree analysis of KOS79 and KOS63-like variants.



Supplementary Figure 1: SplitsTree analysis of KOS79 and KOS63-like variants.

SplitsTree analysis (44, 45, 70) reveals the close relationship among the KOS63-like variants, as compared to either KOS79 or the HSV-1 reference strain 17. Inset (green box) provides an expanded view and labels for the compact section of the network containing KOS63-like strains. Trimmed genome sequences lacking the terminal copies of the large repeats that flank each unique region were excluded, so that these genome regions did not doubly influence the genetic relatedness. Likewise, reiterations set to match those of strain 17 were excluded, so that these did not unduly influence the network (see Methods for details). Genome accession numbers and sources are listed in Table 1.

Supplementary Figure 2: Simplot and BootScan comparison of KOS79 with consensus of the European/North American and Africa-1 clusters.



Supplementary Figure 2: Simplot and BootScan comparison of KOS79 with consensus of the European/North American and Africa-1 clusters.

SimPlot analysis **(A)** of KOS79, in comparison to KOS63, a consensus of the European/North American cluster (strains HF10, 17, McKrae, F, and H129), and a consensus of the Africa-1 cluster (strains E08, E12, E13, E14, and E19) from our prior study (Figure 2) (42). KOS79 is most closely related to the European/North American consensus strain over most of its length, except for a few short regions where it is most similar to the Africa-1 consensus (e.g. between kilobases 45-50 and 97-102) or where no predominant background can be identified (e.g. between kilobases 60-65 and 80-85). These regions may represent recombination events, although whether recent or ancient cannot be known. The corresponding BootScan plot **(B)** shows chi-shaped crossover signals supportive of recombination with an African strain at ~97-102 kb, although two other areas of African similarity have less well formed signs of crossover. Potential crossover events with KOS63 occur at 60-60 kb and 80-85 kb **(B)**, although the SimPlot signals in these areas are not strong **(A)**.

Supplementary Table 1: Proteins varying between each pairwise combination of KOS63-like strains.

Strains	KOS63	KOS_{Davido}	KOS_{Large}	KOS Kinchington	KOS_{Knipe}
KOS_{Davido}	gB (UL27), VP1/2 (UL36), RR1 (UL39), ICP4 (RS1)	-	-	-	-
KOS_{Large}	UL8, UL9, UL13, UL21, gH (UL22), VP1/2 (UL36), US6	UL8, UL9, UL13, UL21, gH (UL22), gB (UL27), VP1/2 (UL36), RR1 (UL39), ICP4 (RS1), gD (US6)	-	-	-
KOS Kinchington	gB (UL27), VP1/2 (UL36), RR1 (UL39)	VP1/2 (UL36), ICP4 (RS1)	UL8, UL9, UL13, UL21, gH (UL22), gB (UL27), VP1/2 (UL36), RR1 (UL39), gD (US6)	-	-
KOS_{Knipe}	gB (UL27), VP1/2 (UL36), RR1 (UL39), ICP34.5 (RL1), ICP0 (RL2)	ICP4 (RS1), VP1/2 (UL36), ICP34.5 (RL1), ICP0 (RL2)	UL8, UL9, UL13, UL21, gH (UL22), gB (UL27), VP1/2 (UL36), RR1 (UL39), ICP34.5 (RL1), ICP0 (RL2), gD (US6)	VP1/2 (UL36), ICP34.5 (RL1), ICP0 (RL2)	-
KOS1.1	UL30, VP1/2 (UL36)	gB (UL27), UL30, VP1/2 (UL36), RR1 (UL39), ICP4 (RS1)	UL8, UL9, UL13, UL21, gH (UL22), UL30, VP1/2 (UL36), gD (US6)	gB (UL27), UL30, VP1/2 (UL36), RR1 (UL39)	gB (UL27), UL30, VP1/2 (UL36), RR1 (UL39), ICP34.5 (RL1), ICP0 (RL2)

Supplementary Table 2: Primers for PCR Validation

Primer Name	Target	Sequence
KOS_UL14_F1	UL14	5'-GGGGAACACTATCTGTCGTTGTTGCAGC-3'
KOS_UL14_R1	UL14	5'-GATCGTCTTGCGGACCAGGAGGAGCAA-3'
KOS_UL14_F2_Seq	UL14	5’-GCGTGAGGGTAAGGATGTG-3’
KOS_UL14_F2.1_Seq	UL14	5’-GCGTGAGGGTGAGGATGTG-3’
KOS_UL14_R2_Seq	UL14	5’-CTGGTCATGTGGCAGCTAA-3’
KOS_UL25_F1	UL25	5'-GTCGATATCGAGCGCCGGTTAC-3'
KOS_UL25_R1	UL25	5'-GGTACAGCAGGTAGAGACACAACAC-3'
KOS_UL25_F2_Seq	UL25	5’-CGGAACGTGCACGAGAT-3’
KOS_UL25_R2_Seq	UL25	5’-CGTCATGAAGGTCTTGGACA-3’