Ancient DNA provides evidence of 27,000-year-old papillomavirus infection and long-term codivergence with rodents

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Abstract
The long-term evolutionary history of many viral lineages is poorly understood. Novel sources of ancient DNA combined with phylogenetic analyses can provide insight into the time scale of virus evolution. Here we report viral sequences from ancient North American packrat middens. We screened samples up to 27,000-years old and found evidence of papillomavirus (PV) infection in Neotoma cinerea (Bushy-tailed packrat). Phylogenetic analysis placed the PV sequences in a clade with other previously published PV sequences isolated from rodents. Concordance between the host and virus tree topologies along with a correlation in branch lengths suggests a shared evolutionary history between rodents and PVs. Based on host divergence times, PVs have likely been circulating in rodents for at least 17 million years. These results have implications for our understanding of PV evolution and for further research with ancient DNA from Neotoma middens.

Key words: paleovirology; papillomavirus; rodent; ancient DNA.

1. Introduction
A major question in viral evolution is the timescale of coevolution between host and virus (Holmes 2009). A pattern of codivergence, or cospeciation, can occur when a host population splits and precipitates a coincident divergence in a pathogen (in this case a virus) infecting the ancestral host population (Haferl and Nadler 1988; Huyse et al. 2005). In cases where viruses are specific to a single host, the phylogeny of the virus becomes largely congruent with that of the host (Jackson and Charleton 2004; Switzer et al. 2005; Katzourakis et al. 2009; Sharp and Simmonds 2011). In contrast, if there is frequent cross-species transmission of viruses across hosts, the phylogenies will not be congruent. Determining the relative impact of these processes during viral diversification is a major question in viral macroevolution (Kitchen et al. 2011).

The Papillomaviridae are double-stranded circular DNA viruses that infect a broad range of vertebrates (de Villiers et al. 2004; Bernard et al. 2010; Rector and Van Ranst 2013). Papillomaviruses (PVs) are important agents of several human and animal cancers (zur Hausen 2002; Moody and Laimins 2010; Rector and Van Ranst 2013; Doorbar et al. 2015). The evolutionary history of PVs is complex, with descriptions in the literature of both strict host-virus codivergence in some taxa and cross-species transmission in others (Ong et al. 1993; Bernard 1994; Gottschling et al. 2007, 2011; Rector et al. 2007; Shah et al. 2010). PVs isolated from birds and turtles form a monophyletic group distinct to those from mammals, but within the mammalian PVs there is no strict pattern of codivergence that would unambiguously indicate an ancient relationship between host and virus. For example, PVs isolated from the same host species are often paraphyletic (García-Pérez et al. 2013, 2014). In the well-studied organism, humans, over 150 distinct PVs have been discovered (de Villiers et al. 2004). One hypothesis for this pattern is that PVs colonized...
new tissue types in ancestral mammals as novel environments like fur evolved (Gottschling et al. 2011). In general, PVs infecting cutaneous and epithelial tissues do not cluster together, which provides some evidence for an ancient radiation event in the primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate primate 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recapitulates the two established rodent families, Cricetidae and Muridae, with strong support (bootstrap value = 100%; Fig. 2). Within Muridae, the genera Rattus and Micromys are highly supported as a monophyletic clade (96% bootstrap support). Finally, the clade containing Mus, Apodemus, and Mastomys is highly supported with bootstrap values of 100%, with the Mus, Apodemus grouping having slightly lower support value of 90%.

The rodent PV phylogeny is largely congruent with the host phylogeny, except for the placement of AsPV and McPV relative to the hosts they were isolated from—Apodemus sylvaticus and Mastomys coucha, respectively. The virus phylogeny also strongly supports the split between viruses isolated from rodents in the family Cricetidae, and rodents in the family Muridae with bootstrap values of 100% for each. Similar to the hosts, the lowest support values are found with the relationship between PVs isolated from Apodemus, Mus, and Mastomys, which have bootstrap support values of 70 and 88%.

Finally, we tested whether there is a relationship between the branch lengths of the congruent host and virus phylogenies. Branch lengths between PVs and their rodent hosts are significantly correlated, suggesting rodents and their PVs share a
similar evolutionary history ($R^2 = 0.52$, $P = 0.012$; Fig. 3). This pattern is expected only if host and virus have a shared evolutionary history. The pattern of codivergence between rodents and their PVs strongly suggests a long-term association, which justifies the calibration of a molecular clock based on host divergence dates. Based on previously published estimates of host divergence times, we estimated the substitution rates using a Bayesian relaxed clock model for rodent PVs and compared them to previously published estimates of feline PV substitution rates. The mean age estimate for the root of this clade of rodent PVs was 17.7 million years ago (95% CI 15.6–19.8 mya). The mean substitution rate estimates for each gene, along with the 95% CIs intervals are shown in Fig. 4. The overall substitution rate for $E_1$, $E_2$, $E_6$, $E_7$, $L_1$, and $L_2$ for rodent PVs was $5.2 \times 10^{-8}$. On average, rodent PVs had substitution rates estimated to be ~2.8 times higher than feline PVs. The genes $E_6$ and $E_7$ from rodent PVs showed higher substitution rates than any of the feline PV genes.

3. Methods

3.1 DNA extraction of Neotoma midden material

The packrat middens analyzed for this study were originally collected in 1979 from a cliff at 1770 m of elevation on the south arm of Poston Butte, just east of Chuar Valley in the Grand Canyon, Arizona (36° 10’ 27” N.; 111° 54’ 5” W) (Cole 1990). They were part of a study that first described the elevational movement of vegetation zones within the Grand Canyon between the Pleistocene and Holocene (Cole 1982). These middens had been preserved in a repository at Northern Arizona University containing several thousand similar deposits from western North America and the Middle East. Because of the vegetation surrounding the deposits at that time, and the inclusion of fossil tooth (RM3) identified as being from Neotoma cf. cinerea (Cole and Mead 1981), it is most likely that the packrat species producing the middens was $N$. cinerea (bushytailed packrat), although another tooth (LM3) was identified as Neotoma lepida (Desert Packrat).

All DNA extractions and reagent preparation were performed in rooms physically separated from any PCR amplification, and the actual DNA extractions were performed in a biosafety cabinet inside of a BSL-2 laboratory. Extraction of rodent samples or PVs had never been performed in the laboratory prior, and rigorous protocols were followed to prevent contamination. During each DNA extraction, a negative control was run alongside the others to ensure that contaminants were not introduced through reagents or other sources. (None of the negative controls yielded PV amplification products.) After cutting introduced through reagents or other sources. (None of the negative controls yielded PV amplification products.) After cutting

3.2 PCR amplification of PV and cytB fragments from midden DNA

Primers specific for rodent PVs and Neotoma cytB were designed from alignments created in house by using sequences downloaded from GenBank. Negative PCR controls were included during all PCR steps and yielded no spurious amplification products. DNA was amplified using two rounds of nested PCR. In the first round, $2 \mu l$ of the DNA extraction was amplified using AmpliTaq Gold 360 DNA polymerase (Thermo Fisher Scientific), in a 50 $\mu l$ reaction volume, with a final concentration of 0.4 $\mu M$ of each primer, 200 $\mu M$ dNTP, 1× AmpliTaq Gold 360
3.3 Phylogenetic analysis of PV/rodent associations

A global analysis of animal PVs was performed to infer the relationship of all described isolated rodent PVs. Gene sequences were downloaded from NCBI (accession numbers available in Supplementary Table S1), and only the most conserved regions of L1, L2, and E1 were used to infer the phylogeny. Due to low sequence identity, nucleotide sequences were translated to amino acids, and a phylogeny was inferred using the LG substitution model with a gamma rate variation distribution in SeaView 4.5.3 using PhyML (Le and Gascuel 2008; Gouy et al. 2010; Guindon et al. 2010). Branch support was calculated with aLRT (Anisimova and Gascuel 2006). The phylogeny of the eight rodent hosts was inferred from publically available sequences downloaded from NCBI GenBank and included four nuclear loci (breast cancer 1 (BRCA1), Growth hormone receptor (GHR), Retinol-binding protein 3 (IRBP), and Recombination activating gene 1 (RAG-1) and one mitochondrial locus (cytB). In a few instances, sequence data were not available for a particular rodent species. For these cases, a sequence from the same genus was used. These cases are noted in the Supplementary Table S1.

Sequences were aligned and trimmed in Geneious 8.1.5 to remove positions in the sequence that contained an ‘N’ (Kearse et al. 2012). Host loci were analyzed as separate partitions to infer the phylogeny. Due to low sequence evolution and 100 bp iterations (Stamatakis 2014). Host loci were analyzed as separate partitions to infer the phylogeny. Due to low sequence evolution and 100 bp iterations (Stamatakis 2014).

For the codivergence analysis, rodent PV sequences included in the analysis were downloaded from GenBank, which had been isolated from R. norvegicus (GQ180114) (Schulz et al. 2009), Micromys minutus (NC_008582) (Van Doorslaer et al. 2007), A. sylvaticus (NC_024893) (Schulz et al. 2012), M. musculus (NC_014326) (Joh et al. 2011), M. coucha (NC_008519) (Amtmann et al. 1984), Phodopus sungorus (HG939559) (Kocjan et al. 2014), and Mesocricetus auratus (NC_022647). Alignments of each coding region were made with MUSCLE, and were then hand aligned to be in-frame in Geneious 8.2.9 (Edgar 2004). To infer the PV tree only the genes E1, E2, L1, and L2 were used due to their relative conservation compared with other genes. Phylogenetically informative segments of the alignment were extracted using GBLOCKS 0.91 bp with default settings keeping codons intact (Castresana 2000). Due to high saturation at the third codon position as determined by DAMBE 6.4.2 (ICC > ICC.c), third codon positions were stripped from the alignment (Xia 2013). The PV phylogeny was inferred by RAxML 8.2.9 with the GTR+gamma model of sequence evolution and 100 bp iterations.

Substitution rates of rodent PVs were calculated with BEAST v1.8.1 (Drummond et al. 2006, 2012). Substitution rates were calculated for each gene as a separate partition for the PV genes E1, E2, E6, E7, L1, and L2. To determine the most appropriate evolutionary model all gene-specific alignments were tested in jModelTest (Posada 2008). Of the models tested, the GTR substitution model had the best fit for each alignment and was implemented with estimated base frequencies and a gamma distributed site heterogeneity model. Three calibration dates were used for the PV phylogeny: the time of the most recent common ancestors (TMRCA) of (1) the rodent subfamily Murinae, (2) the family Cricetidae, and (3) of Mus plus Mastomys, based on previous work that incorporated fossil data to estimate divergence times of different rodent groups (Steppan et al. 2004). Prior probabilities for the TMRCA of the rodent subfamily Murinae were drawn from a normal distribution with a mean of 10.3 million years ago (mya) and a SD of 0.2 mya. For Cricetidae the mean was set to 13.8 mya and SD to 0.69 mya. And for the Mus/Mastomys ancestor, the mean was set to 8.8 mya and SD to 0.3 mya. All other priors were kept at their default settings except the uclde.mean parameter, which was changed to have a gamma distribution with a shape value of 0.01 and a scale of 100. Two independent runs were set-up with 100 million generations sampling the chains every 2000 generations. Log files were examined in Tracer v1.6 to check that ESS values were >200 and to check convergence of the two runs, with 10 million generations discarded as burnin.

4. Discussion

In this article, we describe ancient PV sequences derived from rodent middens, and compare them to other published rodent PVs. We show there is a correspondence in the overall tree topology and relative branch lengths for this lineage of rodent PVs and their hosts, which suggests a long-term shared evolutionary history of codivergence. Although our overall PV phylogeny reveals four independent rodent PV lineages, which does not indicate strict host/pathogen codivergence in the overall PV phylogeny, this particular clade of rodent PVs does fit a pattern of codivergence. Others have noted a pattern of codivergence in some rodent PVs (Schulz et al. 2009). However, this prior work involved fewer lineages, did not compare the differences in branch lengths between host and PVs, and did not use independent host divergence times to estimate a substitution rate. Moreover, we used ancient DNA to provide direct evidence of infection from 27,000 years before present.

One caveat with our analysis is that although a significant relationship exists between the branch lengths of the host and virus phylogenies, and overall there is high concordance between the tree topologies, there is not perfect concordance. Additionally, due to the low number of nodes we did not perform a statistical test of topological concordance between the host and virus phylogenies. Discordance between host and virus trees can arise for a number of reasons, including cross-
species transmission, problems in phylogenetic reconstruction, and recombination (Huyse et al. 2005; Nieberding and Olivier 2007). Our analysis of host relationships among rodents is based on five loci (four nuclear and one mitochondrial), which limits the power to make conclusions regarding the placement of taxa. Additionally, in some cases some loci were not available for certain taxa, so sequences from a closely related taxon were used instead. This may have introduced some noise into the estimates of the host branch lengths (but is not expected to have systematically biased the overall results.) Within Muridae, the lowest support for both the host and virus taxa is within the placement of Mus, Apodemus, and Mastomys. These three species were a part of an rapid radiation that occurred at the basal position of the core Murines, which makes phylogenetic reconstruction difficult (Steppan et al. 2004). Interestingly, phylogenetic studies involving more taxa, more loci, and a supertree approach consistently placed Mus and Mastomys as sister groups, with Apodemus as the outgroup, although in all cases the nodes were poorly supported, demonstrating the difficulty in resolving this rapid radiation (Steppan et al. 2005; Rowe et al. 2008). This placement of taxa differs from our host tree, but if correct would make our PV phylogeny in Fig. 2 completely congruent with the host phylogeny.

Another caveat is the limited number of sequences from this clade of rodent PVs. In total there are eight described sequences in this group isolated from rodents; however, there are over 1,300 species in the superfamily Muridae (Michaux et al. 2001). Thus, our conclusions on codivergence in this group are based on <1% of all rodent species. Greater sampling of divergent rodent lineages for novel PVs is needed in the future.

Phylogenies of rapidly evolving viruses have been shown to recapitulate host movement and population structure over short periods of time (Biek et al. 2006; Thapa et al. 2016). In this paper we add to the building evidence that diversification patterns in relatively slowly evolving DNA virus such as PV can also match host divergences over millions of years. This long-term association has been observed in other DNA viruses. For example, DNA viruses from the family Polyomaviridae have likely been codiverging with their hosts for ~500 million years (Buck et al. 2016), Hepadnaviridae for ~430 million years (Lauber et al. 2017), and Baculoviridae for ~310 million years (Thézé et al. 2011). Our description here of a case of apparent codivergence between some rodent groups and their PVs, along with previous evidence of codivergence of PVs in felines (Rector et al. 2007). We estimated an average substitution rate for 6 genes in rodent PVs of 5.2 × 10^-8. Our estimate of the root of the rodent PV phylogeny was 17.7 million years. This represents the TMRCA of Muridae and Cricetidae and is younger than the 24.7 million years estimated from host phylogenies and the fossil record (Michaux et al. 2001; Steppean et al. 2004). Systematic underestimation of substitution rates in deep portions of viral phylogenetic trees has been noted in several viral lineages, leading to underestimation of TMRCA’s (Ho et al. 2007; Wertheim and Kosakovsky Pond 2011). Accordingly, it is not surprising that our analysis also underestimated the age of the deepest node, albeit only slightly.

Our ancient PV sequences are novel for two reasons. First, although a large number of ancient viruses have been described, this represents the oldest PV sequence that we are aware of. Second, this is the first description of ancient viral DNA from an animal midden. Ancient rodent material has been used, but never middens made specifically by rodents in the genus Neotoma (Kuch et al. 2002; Murray et al. 2012). The fact we were able to get relatively long continuous stretches of DNA (414 bp) from a 27,000-year-old sample is somewhat surprising. Typically, DNA is thought to be best preserved at cold temperatures. In our case, middens were preserved in dry caves within the Grand Canyon. Ancient Neotoma middens are hard dense structures of dried urine, plant material, and fecal pellets. There may be something specific about the biochemical properties of Neotoma middens that enable long-term preservation of relatively long stretches of DNA. Hundreds of middens spanning from the present to over 45,000-years old have been collected and studied for plant macrofossils across western North America. Since they contain host and pathogen DNA, and likely plant DNA, they represent very promising resources to study genetic changes over tens of thousands of years across different environments in western North America, and clearly can be an important window into not just hosts but also viral pathogens.

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**Data availability**

Sequences were deposited into Genbank with accession numbers MH136585-MH136587.

**Supplementary data**

Supplementary data are available at Virus Evolution online.

**Conflict of interest**: None declared.

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