

# Monkeypox viruses circulate in distantly-related small mammal species in the Democratic Republic of the Congo

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## Abstract

We determined near complete and complete monkeypox virus genomes in a shrew (*Crocidura littoralis*), two squirrels (*Funisciurus anerythrus*, *Funisciurus bayonii*), and produced shorter sequences from two rats (*Stochomys longicaudatus*, *Cricetomys* sp. 2) originating from the Democratic Republic of the Congo. This suggests that a number of rodents besides squirrels (families Muridae and Nesomyidae) and shrews (order Eulipotyphla) are potential monkeypox virus reservoirs.

## Main Text

Human monkeypox is a zoonotic disease caused by monkeypox virus (MPXV), a member of the *Orthopoxvirus* (OPXV) genus in the *Poxviridae* family. The clinical presentation generally resembles smallpox: after a mean incubation time of 8 days (4-14 days), most patients first experience fever and a few days later a characteristic rash<sup>1,2</sup>. Case fatality rate is usually low in adults, but can reach well over 10% in children. The virus naturally occurs in Africa where two genetic clades have been discerned: a West African clade (MPXV-WA) that is only found in countries situated west of Cameroon, and a Congo Basin (MPXV-CB) clade only observed in Central Africa<sup>1-3</sup>. While the MPXV-CB was generally considered to be more virulent and transmissible in humans (based on a low sample size)<sup>4</sup>, the recent Nigerian outbreak (2017-2018) provided evidence of increased virulence of MPXV-WA<sup>5</sup>. The increasing incidence and human-to-human transmission for both strains in endemic areas highlight the need for a better understanding of the eco-epidemiological drivers of the disease<sup>6</sup>.

The presumptive animal source of primary human infections remains unidentified and the virus' natural reservoir cryptic<sup>7</sup>. MPXV has only been isolated from three African endemic wild mammals: in the Democratic Republic of the Congo (DRC) from a Thomas's rope squirrel (*Funisciurus anerythrus*), and in the Taï National Park in Côte d'Ivoire from a sooty mangabey (*Cercocebus atys atys*) and western chimpanzees (*Pan troglodytes verus*)<sup>8-10</sup>. Whereas primates are assumed to be incidental hosts, different species of rodents are considered to be potential reservoirs of the virus (such as *Cricetomys*, *Graphiurus*, *Funisciurus* and *Heliosciurus* sp). However, empirical data to support these claims is scarce and mainly based on serological data or DNA amplicons without confirmation by sequencing<sup>11-15</sup>.

Since 2010 we have been monitoring the biodiversity of small mammals and their pathogens in several areas in the Ituri and Tshopo provinces of the DRC. A subset ( $n=256$ ) of samples (random selection of samples that were completely submerged in ethanol, Supplementary excel file: 'small\_mammals\_MPXV'), including tissues collected from rodents ( $n=97$ ), shrews ( $n=93$ ) and bats ( $n=66$ ) over a period of six years, was tested for the presence of MPXV DNA using two PCR assays targeting different viral genes (P4A and haemagglutinin) and positives were confirmed by Sanger sequencing<sup>16,17</sup>. Tissues from individuals representing four rodent species (*Funisciurus anerythrus*, *Funisciurus bayonii*, *Stochomys longicaudatus*, *Cricetomys* sp. 2 *sensu* Olayemi et al. 2012) and one shrew (*Crocidura littoralis*) contained MPXV DNA. By applying in-solution hybridization capture and high-throughput sequencing<sup>10</sup>, we successfully

retrieved two complete MPXV genomes from *F. anerythrus* and *C. littoralis*, and one partial genome (59%) from *F. bayonii*. Therefore, MPXV appears to naturally circulate in multiple, distantly-related rodent species from at least three different families (Sciuridae, Muridae and Nesomyidae), as well as in shrews (order Eulipotyphla). While some of these detection events may reflect dead-end infections, they are compatible with the hypothesis that MPXV has a broad host range and possibly a complex multi-host ecology<sup>18</sup>. The ability to infect multiple host species is also known for other OPXVs, including the better-studied cowpox virus (CPXV) which infects at least three rodent species (field voles, bank voles and wood mice) in the UK, sometimes leading to spillovers into bovids<sup>14</sup>. Because OPXVs are relatively stable in the environment and able to infect susceptible hosts via multiple routes (respiratory, mucosal and parenteral), cross-species transmission of OPXVs is likely facilitated by indirect transmission and might happen frequently between species<sup>18</sup>.

Although MPXV is able to infect multiple hosts, it does not necessarily imply that all hosts contribute to viral persistence. In fact, it is often suggested that the majority of transmission events and pathogen persistence is associated to a key host species<sup>19</sup>. Consequently, much of the apparent complexity of such multi-host systems could be simplified by focussing on one or two key hosts within the community. The key position of these hosts may be the outcome of separate processes such as infection prevalence, population density and transmissibility<sup>19</sup>. For example, while CPXV can be maintained in wood mice populations in the absence of other mammal species (potentially due to their high population abundance), its transmission in bank voles populations largely depends on the presence of wood mice or field voles in the wildlife community<sup>20</sup>. It is possible that the maintenance of MPXV also depends on one or two primary animal reservoirs and that other species serve as auxiliary hosts. The results reported here adds to the limited evidence pointing at *Funisciurus* squirrels as potential primary reservoirs for MPXV. Squirrels from this genus show the highest seroprevalence of all rodents and they have allowed for the isolation of the virus and now for its whole genome sequencing from multiple individuals<sup>11,12,15</sup>. Within this genus, *F. anerythrus* maybe sticks out as a particularly plausible primary host due to its social behaviour (more interaction with conspecifics) and higher densities than any other species of African tree squirrel, both characteristics facilitating viral intra-species transmission<sup>21</sup>.

Nevertheless, the fact that MPXV was first isolated from a *Funisciurus* squirrel might have biased our view on MPXV ecology, as most follow-up studies targeted squirrels and potentially neglected other animal orders, such as shrews or bats. Similarly, the multimammate mouse was for a long-time assigned to be the sole Lassa arenavirus (LASV) reservoir, as the virus was first isolated from this rodent species which is highly abundant around houses in areas where the disease is endemic. However, recent studies also detected LASV in rodent communities even in the absence of the multimammate mouse<sup>22</sup>. This suggests that the concept of a 'primary reservoir' might often depend on the context. For example, it might be that MPXV transmission is maintained by *Funisciurus* squirrels in areas where these rodents are abundant, while other species are needed for viral persistence in areas where *Funisciurus* squirrels are less abundant, or absent. Additional field studies targeting a larger variety of mammals and following populations over time are necessary to confirm the hypothesis that *Funisciurus* squirrels are indeed the

primary MPXV reservoirs, or to support the hypothesis of ‘combined maintenance’ in the wildlife community.

Maximum likelihood (ML) and Bayesian phylogenetic analyses revealed that the MPXVs found in the red squirrel (*F. anerythrus*) and shrew are closely related to each other and fall within the diversity of MPXV strains found in humans in Central Africa (**Fig 1**), with the time to the most recent common ancestor (tMRCA) with the closest published genomes dated to 1959. Similarly, ML analyses performed on the partial MPXV genome found in the *F. bayonii* showed that it clusters with other MPXV-CB strains (Supplementary Fig. 1). The presence of the new animal-derived sequences provided an opportunity to reassess the standing hypothesis that MPXV diversification essentially reflects ancient vicariance events caused by climate-induced forest-habitat contractions, such as the opening of the Dahomey gap (4,500BP) or the Last Glacial Maximum (21,000BP)<sup>2</sup>. We found, however, that such diversification was too recent to be compatible with these events, with the MPXV tree root being about 600 years-old in the best model. However, given the relatively modest divergence between MPXV-WA and MPXV-CB, we do not expect this would affect the estimated evolutionary time scale of MPXV to such an extent that it would become compatible with key climate factors (4,000-25,000BP) as proposed in Nakazawa et al<sup>2</sup>. Rather, we consider that it is more likely that the geographical isolation of the two clades has occurred more recently (e.g. during the Medieval Warm Period; 700-1,100BP)<sup>23</sup> or that alternative evolutionary mechanisms were at play. The WA and CB MPXV viruses may for example circulate in distinct hosts communities, with MPXV-CB depending on hosts strictly endemic to Central Africa for its long-term persistence.

In conclusion, the presence of MPXV DNA in diverse forest dwelling rodents and shrews sheds a new light on the ecology of MPXVs. This finding calls for increased surveillance in a wider range of African mammals to reveal the host range and distribution of MPXV, and possibly to discern whether different animal groups host distinct MPXV strains with distinct virulence.

## Methods

Small mammals were trapped at different locations in Ituri and Tshopo provinces of the DRC between 2010 and 2016 using Sherman live traps, pit falls and mist nets (2074 rodents, 992 insectivores and 525 bats). Captured animals were humanely euthanised (using isoflurane) and necropsied *in situ*. Tissues samples (kidney, liver and spleen) were stored in 75% ethanol and shipped to the University of Antwerp (Belgium), where samples were stored in the dark at room temperature. DNA was extracted on a mixture of the different organs using the NucleoSpin tissue kit (Machery Nagel, Düren, Germany) according to the manufacturer’s instructions. Species identification was done based on morphological measurements and confirmed with mitochondrial DNA cytochrome *b* sequencing using L7 and H6 primers and 16S rRNA for shrews. The sequences obtained were compared with known sequences from GenBank and the *African Mammalia* database (<http://projects.biodiversity.be/africanmammalia>).

For MPXV screening, a TaqMan Real-time PCR targeting the OPXV P4A gene of all orthopoxviruses was performed *in duplo* on a subset of samples (see supplementary data: excel 1)<sup>17</sup>. Positives, were confirmed using a conventional PCR targeting a 270bp fragment of the MPXV haemagglutinin (HA) gene<sup>16</sup>, Sanger sequenced and shipped to Robert-Koch Institute for whole genome sequencing as described in Patrono et al<sup>10</sup>. In short, Illumina-compatible libraries were built from fragmented DNA (250ng DNA in 50 µl EDTA TE buffer) using the NEBNext Ultra II kit and quantified using the KAPA HiFi library quantification kit. To increase the relative amount of MPXV DNA in the libraries with respect to background DNA, two rounds of 24h hybridization capture at 65°C were performed using 2-fold tiling 80-mer RNA baits (MYBaits®) designed to target orthopoxviruses<sup>10</sup>. The enriched pool was diluted to 4nM and sequenced on a NextSeq platform (2x150 bp). Raw NGS reads were processed following the pipeline described in Patrono et al<sup>10</sup> with an additional step to merge paired reads using the ClipAndMerge tool from Eager<sup>24</sup>. Sequences KJ642616 and JX878411 were used for reference-based mapping to either a West African of Congo Basin clade MPXV genome, respectively. Since the two ends of MPXV genomes are inverted terminal repeats (ITRs) that contain many repetitive regions and indels that can lead to difficult or erroneous assembly, the right ITR was removed from both reference sequences (from position 190375 and 191567 in the KJ642616 and JX878411 sequence, respectively). Consensus sequences were called at positions covered by at least 20 reads (95% agreement) for MPXV from *F. anerythrus* and *C. littoralis*, whereas for *F. bayonii* calling was set to at least 2 reads (less on-target reads were obtained for this genome). For phylogenetic analyses, we downloaded all publicly available MPXV complete genomes from the National Center for Biotechnology Information (NCBI) database ( $n=61$ ) and added the two complete genome sequences herein generated (accession numbers: MT724770 and MT724772). Genomes ( $n=63$ ) were aligned using MAFFT v7 and conserved blocks were selected using GBlocks in Seaview v452, resulting in an alignment of 178,345 positions (see supplementary file: GBlocks). Upon removal of sites containing ambiguities ( $n=2618$ ), the alignment was reduced to variable sites ( $n=2176$ ) by stripping all identical sites ( $n=173,551$ ) and collapsed to unique sequences ( $n=50$ ) with the online tool FaBox v1.453. We ran recombination analyses with RDP4<sup>25</sup> and removed minor recombining fragments. We then ran exploratory phylogenetic analyses, to determine whether a temporal signal was likely to exist in this dataset. For this we used PhyML v.3 with smart model selection<sup>26</sup> (GTR) to obtain an unrooted maximum likelihood (ML) tree (Supplementary Fig 1). A first analysis of the resulting tree in TempEst<sup>27</sup> identified one outlier sequence (AF380338) that showed a suspiciously long branch; upon removal of this sequence a moderate temporal signal was detected (Rsq=0.33).

We went on by running formal molecular clock analyses in a Bayesian framework using BEAST v1.10.4<sup>28</sup>. We assumed a coalescent tree shape prior (constant population size), performed multiple Bayesian Markov chain Monte Carlo (BMCMC) runs and checked multiple run convergence as well as appropriate sampling of the posterior (effective sample size > 200) using Tracer v1.7<sup>29</sup>. We first wanted to confirm the presence of a temporal signal. To do this, we applied a recently suggested approach whereby for the same dataset for which tip dates are available the fits of two models are compared, one of which is informed by these tip dates (heterochronous model) while the other is not (isochronous

model)<sup>30</sup>. We estimated model marginal likelihoods using a generalized stepping stone procedure<sup>31</sup> and compared models using Bayes factors (BF); we considered support for one of the competing models was decisive where  $2\ln BF > 10$ . We implemented the two most extreme forms of molecular clocks, i.e. a strict molecular clock where a single substitution rate is applied across the entire phylogeny and an uncorrelated relaxed clock where the substitution rate is allowed to vary from branch to branch and is drawn from a lognormal distribution. BF comparison showed the presence of a temporal signal under both models (**Table S1**). Maximum clade credibility (MCC) trees were generated using TreeAnnotator v.1.10.4, using combined tree files generated with LogCombiner v1.10.4 (both softwares are distributed with BEAST)<sup>28</sup>. ITol v5 was used for both tree visualization (<https://itol.embl.de/>). Branch robustness was assessed through posterior probabilities.

For the *F. bayonii* partial genome sequence, we ran ML analyses in PhyML as described above (Supplementary Fig 2).

## Declarations

### Data availability statement

Sequence data that support the findings of this study have been deposited in GenBank with the accession codes: MT724770- MT724772 and as a supplementary file, together with the Illumina reads (SF2).

### Ethical statement and transfer of samples

There is no IACUC linked to the sampling of animals because there was no dedicated animal ethical committee in the DRC at the time of sampling. However, the research protocols of the different projects (COBIMFO, VLIR UOS, VLIR IS) have been submitted and approved by the owner (University of Antwerp, Belgium) and addressee (the Institute of Public Health in Kinshasa) institutional scientific boards. The research permits and authorizations were granted by the University of Kisangani Biodiversity Surveillance Center (CSB [Centre de Surveillance de la Biodiversité] – in French) which has the scientific authority to do so. All “movement of personal” (called “ordre de Mission”) were signed and approved by the local authorities at each field site. Material transfer agreements (MTA) were issued by the University of Kisangani, Biodiversity Surveillance Center (CSB [Centre de Surveillance de la Biodiversité] – in French). None of the animal samples are listed on the CITES Red List of protected species nor on the national list of protected fauna of the DRC ; hence no CITES permit was needed prior to shipping. All animal were captured using live traps and humanely euthanized (isofluorane) following the 2013 AVMA Guidelines for the Euthanasia of Animals and Sikes and Gannon 2007 (J Mammal. 88:809–23).

### **Author contributions**

Conceived the study: JM, AL, LVP, SC, EV and HL. Wrote the paper: JM, AL, LVP, SC. EV. Performed the field work: AL, PB, PM, CM, SN, FvP. Performed the Laboratory work: JM, LP, RvV, NvH, FvP and SG. Performed

the phylogenetic analyses: LVP and SC. Supervised field and laboratory work: AL, JJ-MT, MM, EB, SC, FL, HL, EV. All authors read and approved the final manuscript.

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## Figures

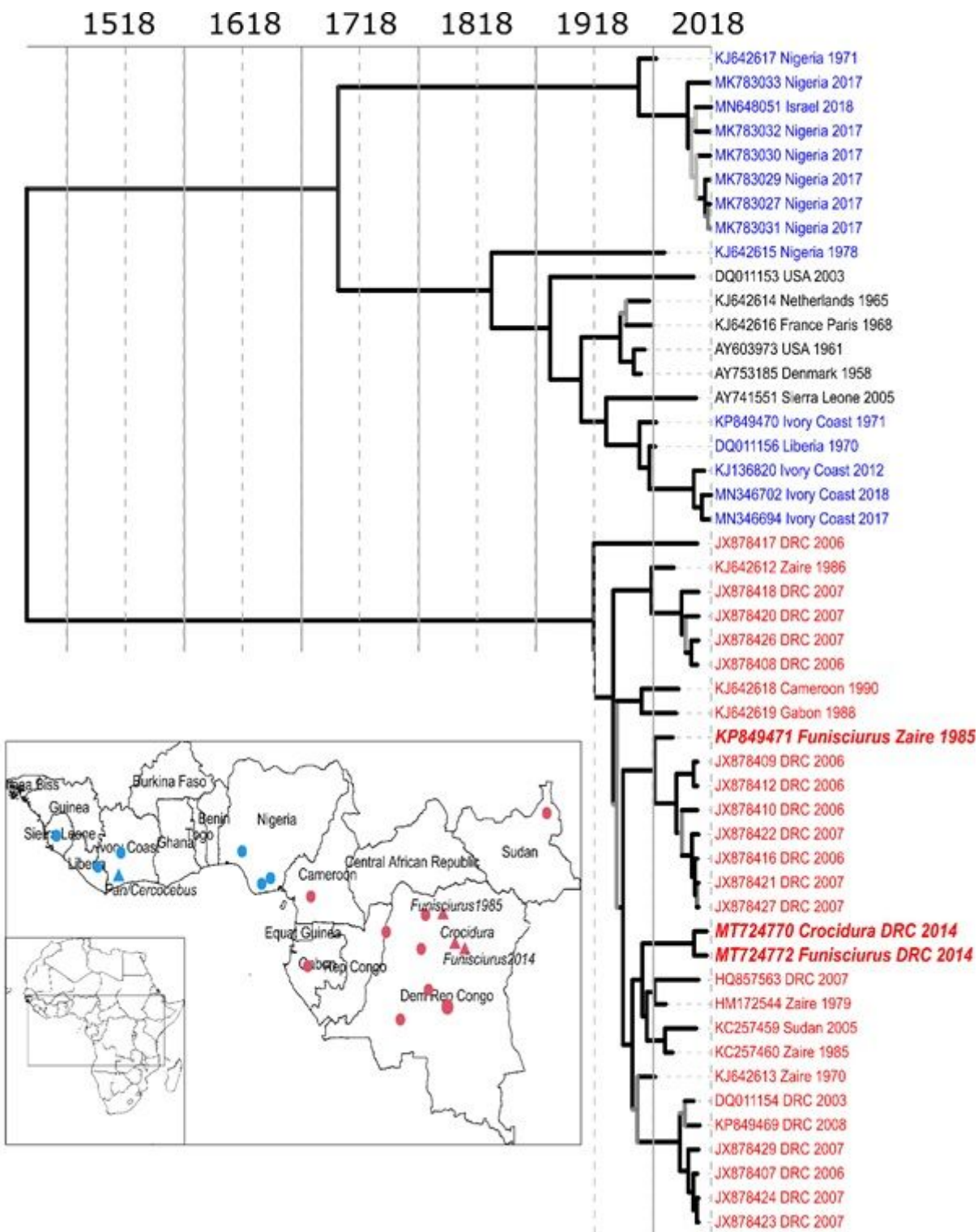


Figure 1

Chronogram derived from an alignment of monkeypox whole genome sequences and map of Africa to show sequence origin based on a molecular clock analysis. Sequences derived from specimens from the Congo Basin are in red, from West Africa in blue, the USA and Europe in black and wildlife derived sequences in bold italic. Human MPXV sequences are represented as dots on the map and animal sequences as triangles. The inner branch colours represent posterior probabilities (grey is <0.95; black is  $\geq 0.95$ ). Note: The designations employed and the presentation of the material on this map do not imply

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