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A multidisciplinary approach to identify the mammal reservoir species of Monkeypox virus in Africa

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1. Introduction

Monkeypox, or mpox, is an emerging infectious zoonotic disease endemic to the rainforests of Central and West Africa, which manifests itself as fever, rash, and swollen lymph nodes. It is caused by the Monkeypox virus (MPXV), which belongs to the family Poxviridae and possesses a double-stranded DNA genome of 197 kb. The first human infection was detected in 1970 in a young boy from Bokenda in the Democratic Republic of Congo [1]. Ever since the disease has been reported in nine African countries from Central Africa (Cameroon, Central African Republic, Democratic Republic of Congo, Gabon, and Republic of the Congo) and West Africa (Côte d'Ivoire, Liberia, Nigeria, and Sierra Leone), with some exportations to America, Asia, and Europe between 2003 and 2021 [2–5]. More recently, more than 80,000 cases have been reported in 2022 worldwide, during an epidemic involving mostly men who have sex with men [6,7].

2. Identifying the most probable mammal reservoir hosts for MPXV based on ecological niche comparisons

Although the MPXV reservoir has not yet been identified, several lines of evidence point to mammal species endemic to African rainforests of West and Central Africa. First, MPXV belongs to the genus *Orthopoxvirus* (OPXV), a genus exclusive to mammals [8,9]. Second, most human mpox cases have been reported in rainforests of Central and West Africa, or travellers from these regions [2–5,10–13]. Third, the geographic distribution of MPXV has been inferred with ecological niche modelling (ENM) methods, and the results have shown that the virus could be found in all rainforests of West and Central Africa [13–16]. Fourth, phylogenetic studies based on complete MPXV genomes have revealed a strong geographic structure [14,17,18]: viruses from Central Africa (Gabon, Cameroon, CAR, Republic of Congo, and DRC; clade I) are divergent from those from West Africa; and the latter can be separated into two geographic subgroups, one including viruses from Sierra Leone, Liberia, Côte d'Ivoire and Ghana (clade IIa), and the other including viruses from Nigeria (clade IIb). These results suggest that the animal reservoir populations have been genetically isolated from each other for many generations in three separate rainforest blocks, including the Upper and Lower Guinean forests in West Africa, and the Congo

Basin in Central Africa (see map in Figure 1). We hypothesized therefore that the MPXV reservoir is represented by one or several rainforest mammal species with a geographic distribution very similar to that of MPXV.

In our first article submitted in January to the journal *Viruses* [19], we therefore explored this hypothesis using a four-step approach: (i) we provide the full list of mammal genera and species previously identified as MPXV natural hosts in Africa; (ii) we predict the geographic distributions (or ecological niche) of all species of these genera based on georeferenced specimens catalogued in museum collection databases and ENM methods; (iii) we reconstruct the ecological niche of MPXV using reliable data on human index cases and MPXV sequences available for georeferenced wild animals; and (iv) we make statistical overlap comparisons between the ecological niches of mammals and MPXV in order to identify the most probable mammalian reservoir(s).

The results of this study confirmed that the MPXV niche covers three African rainforests, the Congo Basin and Upper and Lower Guinean forests (Figures 1A and 1B). Our MPXV niche and recent phylogeographic studies on MPXV [14,17,18] suggest that two biogeographic barriers have isolated mammalian populations of the MPXV reservoir host: on the one hand, the Volta River and Dahomey Gap between the Upper Guinean forests (UGF) and Lower Guin-ean forests (LGF) in West Africa, and on the other hand, the Sanaga River between LGF and the Congo Basin.

Our analyses revealed that the four mammal species showing the best niche overlap with MPXV are all arboreal rodents, including three squirrels, *Funisciurus anerythrus* (Figure 1C), *Funisciurus pyrropus* (Figure 1E), and *Heliosciurus rufobrachium* (Figure 1F) and *Graphiurus lorraineus* (Figure 1D). The niche of *Funisciurus anerythrus* shows the best overlap (using both D and I metrics) with that of MPXV, suggesting that the Thomas's rope squirrel could be the main MPXV reservoir.

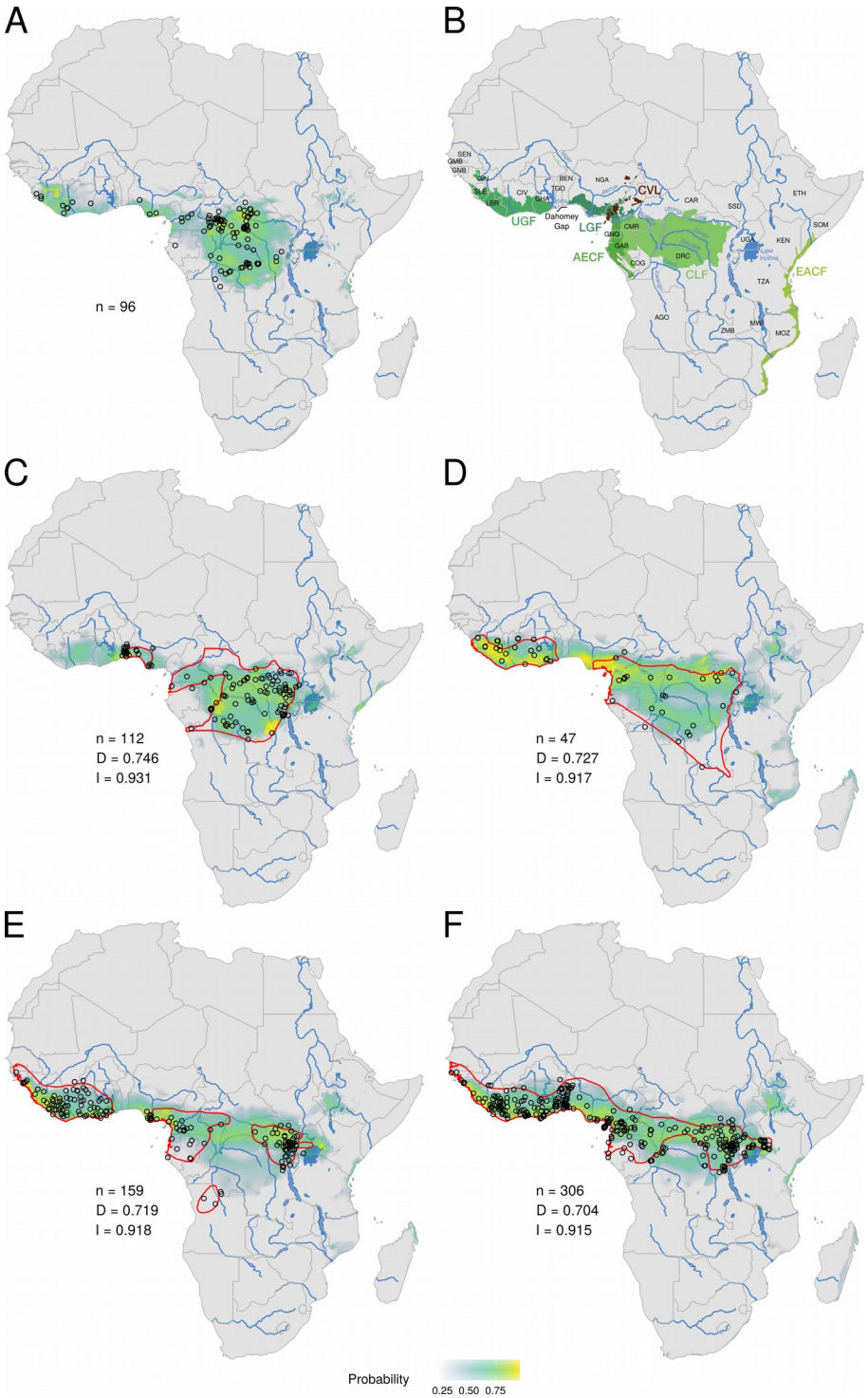

Figure 1. Ecological niches of Monkeypox virus (A) and the four mammal species showing the best overlap with it: *Funisciurus anerythrus* (C), *Graphiurus lorraineus* (D), *Funisciurus pyrropus* (E), and *Heliosciurus rufobrachium* (F). Black circles indicate localities used to build the distribution model. The probabilities of occurrence are highlighted using different colours: blue grey for probabilities < 0.5; turquoise green for 0.5 < p < 0.75; yellowish green for 0.75 < p < 0.9; and yellow for p > 0.9. The red line is the IUCN distribution of the species [86]. At the left of the maps are indicated the number of occurrence records (n) used to infer the ecological niche and the Schoener's D and Hellinger's I values summarizing niche overlap between mammal species and MPXV. For convenience, we have included a map (B) showing the major biogeographic barriers, such as the Dahomey gap, rivers and Cameroon volcanic line (CVL), and African rainforests, including the Upper Guinean forests (UGF) and Lower Guin-ean forests (LGF) in West Africa, the Atlantic Equatorial coastal forests (AECF) and Congolian lowland forests (CLF) in Central Afri-ca, and the Eastern African Coastal Forests (EACF) in East Africa.

3. Taxonomy and phylogeny of African squirrels

Interestingly, *Funisciurus anerythrus* is the only species from which MPXV was isolated [20,21] and fully sequenced by two independent teams in different provinces of northern DRC [14,22]. In addition, two small fragments of MPXV were amplified in museum specimens from five *Funisciurus* species, all collected in rainforests of the Congo Basin: *Funisciurus anerythrus* (45 out of 362 specimens tested; 12,4%), *Funisciurus carruthersi* (3 of out 109 specimens tested; 2.8%), *Funisciurus congicus* (32 out of 239 specimens tested; 13.4%), *Funisciurus lemniscatus* (5 of out 82 specimens tested; 6.1%), and *Funisciurus pyrropus* (8 of out 201 specimens tested; 4.0%) [23]. These data suggest the MPXV reservoir could contain not just one species but several species of *Funisciurus*. In the Congo Basin, however, *Funisciurus anerythrus* is sympatric with the four other species, including *Funisciurus pyrropus* and the three species endemic to Central Africa, *Funisciurus carruthersi*, *Funisciurus congicus*, and *Funisciurus lemniscatus*. As a consequence, it can be hypothesized that *Funisciurus anerythrus* is indeed the reservoir host species, which can contaminate frequently other arboreal species, such as squirrels and monkeys (secondary hosts), due to regular contacts (direct or indirect) in forest trees.

To further investigate this hypothesis, two complementary studies need to be conducted: (i) *Funisciurus* squirrels caught in future field surveys in African forests and those currently housed in museum's mammal collections should be systematically tested for the presence of MPXV; and (ii) mitochondrial and nuclear genes should be sequenced on georeferenced *Funisciurus* specimens to solve some taxonomic problems involving *Funisciurus anerythrus*, *Funisciurus pyrropus* and *Funisciurus substriatus* and to compare the phylogeography of *Funisciurus anerythrus* with that already available for MPXV [18].

Before studying the comparative phylogeography of *Funisciurus* species, we quickly realized that it was necessary to better understand the taxonomy and phylogeny of squirrel species living in the rainforests of Africa. According to recent taxonomic classifications [24], all these species are ranged into six genera of the tribe Protoxerini: *Epixerus* (1 species), *Funisciurus* (10 species), *Heliosciurus* (6 species), *Myosciurus* (1 species), *Paraxerus* (11 species), and *Protoxerus* (2 species). There are currently only five DNA sequences of the mitochondrial gene of the first subunit of the cytochrome c oxydase (CO1), which has been

chosen as the barcode of life for the molecular taxonomy of animal species [25]. All these data belong to only one genus, i.e. *Heliosciurus*, which prevents any study of molecular taxonomy on Protoxerini. As a result, the phylogeny of this group remains totally elusive.

Last years, we collected muscle or skin samples from Protoxerini in seven international institutions (Muséum national d'Histoire naturelle, France; Centre de Biologie pour la Gestion des Populations, France; Institute of Vertebrate Biology, Czech Republic; Museum Koenig Bonn, Germany; Field Museum, USA; Museum of Vertebrate Zoology, USA; American Museum of Natural History, USA) and we systematically sequenced the CO1 barcode fragment. Our phylogenetic results based on these mitochondrial DNA sequences revealed many misidentifications at both generic and species levels among museum specimens of Protoxerini squirrels, including several taxonomic issues between *Funisciurus anerythrus* and *Funisciurus pyrropus.*

To better understand the phylogeny of Protoxerini, we decided to sequence the full mitochondrial and nuclear genomes for a selection of samples. Eight samples were selected in 2021 for whole genome sequencing, including three geographic lineages of *Funisciurus anerythrus* (Central African Republic, Cameroon, and Côte d'Ivoire), *Funisciurus leucogenys*, *Funisciurus lemniscatus*, *Heliosciurus rufobrachium*, *Paraxerus lucifer* and *Protoxerus strangeri*. In 2022, we added six samples of *Funisciurus anerythrus* (Benin, Côte d'Ivoire, Democartic Republic of Congo, Liberia, and Mali), one *Funisicurus carruthersi*, and one *Funisciurus pyrropus*.

By this way, we reconstructed the phylogeny of Protoxerini based on an alignment of the complete mitochondrial genome. The mitochondrial tree of Figure 2A shows that the two species *Funiscirus pyrropus* and *Funiscirus leucogenys* are closely related to *Funisciurus anerythrus*, and that a strong phylogeographic structure is found within *Funisciurus anerythrus*, with a basal dichotomy separating populations from West Africa from those of Central Africa, and the existence of four geographic lineages corresponding to the Upper Guinean forests (samples highlighted in red), the Dahomey Gap (the sample from Benin in orange), the Lower Guinean forests (the sample from Cameroon in blue) and the Congo Basin (samples from CAR and DRC).

However, many previous phylogenetic studies have shown that the mitochondrial tree may be discordant with the species tree because females and males have usually different dispersal behaviours (female philopatry versus male dispersal) and because interspecific

hybrid females are generally fertile, whereas hybrid males are often sterile (Haldane's rule), facilitating mitochondrial introgression between closely related species [26]. Therefore, we extracted 99 nuclear exons from our whole genome sequencing datasets to make phylogenetic comparisons with the mitochondrial tree. The nuclear tree of Figure 2B shows that it is highly similar to the mitochondrial tree of Figure 2A. The sole topological difference concerns interrelationships between *Funisciurus anerythrus* from Côte d'Ivoire, Liberia, and Mali. The comparative phylogeography between MPXV and *Funisciurus anerythrus* (Figure 2) confirms therefore the existence of different geographic lineages (West Africa vezrsus Central Africa; Cameroon versus CAR + RDC) providing additional support for a key role of *Funisciurus anerythrus* as reservoir host of the MPXV.

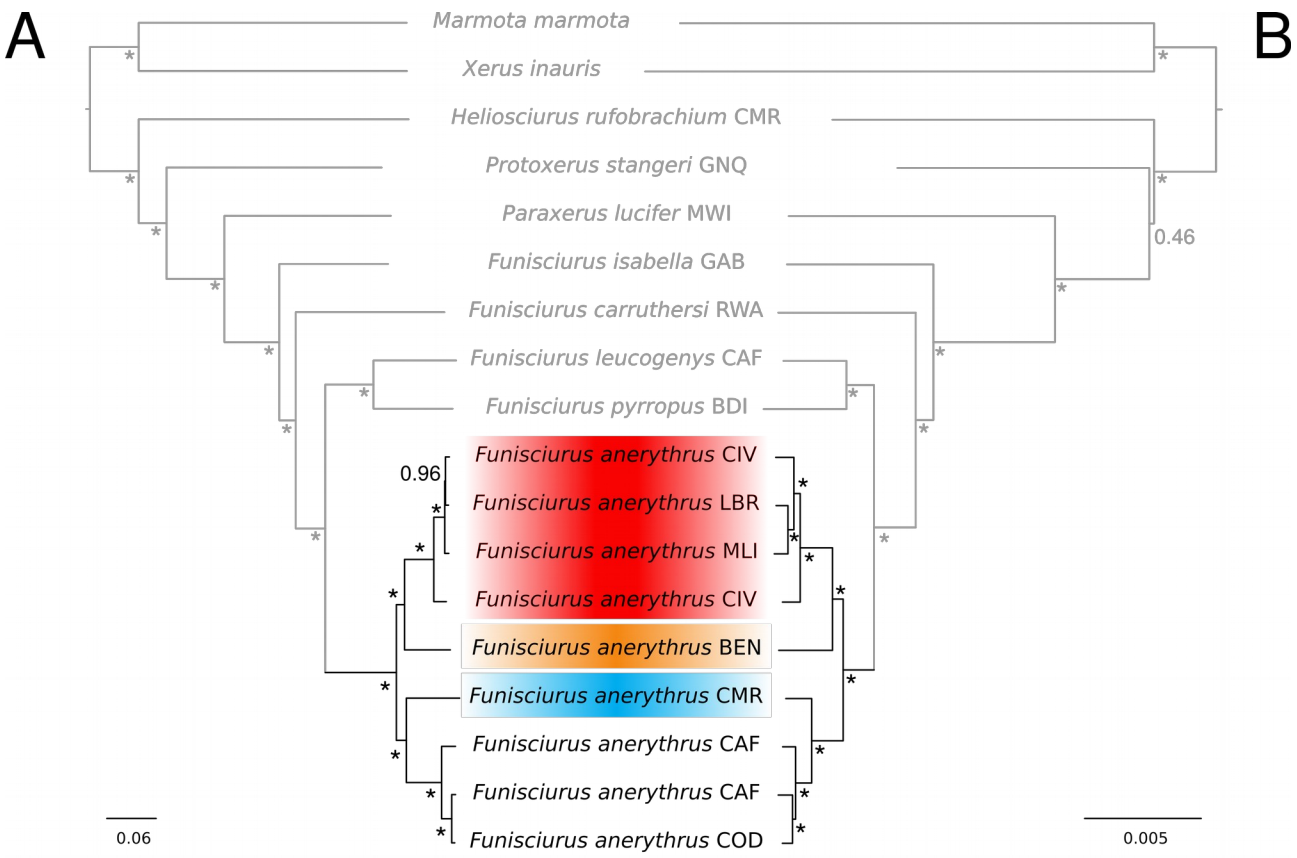

Figure 2. Tree resulting from Bayesian analysis of the complete mitochondrial genome (A) and a concatenation of 99 nuclear exons (B). The mitochondrial tree was reconstructed by the Bayesian method (GTR+I+G model) from an alignment of 18 taxa and 16,812 nucleotides. The nuclear tree was reconstructed by the Bayesian method (GTR+I+G model) from an alignment of 18 taxa and 148,293 nucleotides. The posterior probability is indicated on the nodes. The stars correspond to a posterior probability of 1.

Although these preliminary results need to be improved by including additional squirrel populations in the analyses, they will allow us to get better insights into the co-evolution of MPXV with its reservoir, as well as to have a better understanding of the origin of the two highly divergent geographic lineages of MPXV, both being associated in Central and West Africa with the emergence of mpox disease in humans. This study will be the main focus of this year, which corresponds to the second year of the PhD.

4. References

1. Ladnyj, I.D.; Ziegler, P.; Kima, E. A Human Infection Caused by Monkeypox Virus in Basankusu Territory, Democratic Republic of the Congo. *Bull. World Health Organ.* **1972**, *46*, 593–597.
2. Centers for Disease Control and Prevention (CDC) Multistate Outbreak of Monkeypox–Illinois, Indiana, and Wisconsin, 2003. *Morb. Mortal. Wkly. Rep.* **2003**, *52*, 537–540.
3. Damon, I.K.; Roth, C.E.; Chowdhary, V. Discovery of Monkeypox in Sudan. *N. Engl. J. Med.* **2006**, *355*, 962–963, doi:10.1056/NEJMc060792.
4. Mauldin, M.R.; McCollum, A.M.; Nakazawa, Y.J.; Mandra, A.; Whitehouse, E.R.; Davidson, W.; Zhao, H.; Gao, J.; Li, Y.; Doty, J.; et al. Exportation of Monkeypox Virus from the African Continent. *J. Infect. Dis.* **2020**, *225*, 1367–1376, doi:10.1093/infdis/jiaa559.
5. Sarwar, S.; Maskey, U.; Thada, P.K.; Mustansir, M.; Sarfraz, A.; Sarfraz, Z. Re-Emergence of Monkeypox amidst Delta Variant Concerns: A Point of Contention for Public Health Virology? *J. Med. Virol.* **2022**, *94*, 805–806, doi:10.1002/jmv.27306.
6. Adalja, A.; Inglesby, T. A Novel International Monkeypox Outbreak. *Ann. Intern. Med.* **2022**, *175*, 1175–1176, doi:10.7326/M22-1581.
7. Gessain, A.; Nakoune, E.; Yazdanpanah, Y. Monkeypox. *N. Engl. J. Med.* **2022**, doi:10.1056/NEJMra2208860.
8. Hendrickson, R.C.; Wang, C.; Hatcher, E.L.; Lefkowitz, E.J. Orthopoxvirus Genome Evolution: The Role of Gene Loss. *Viruses* **2010**, *2*, 1933–1967, doi:10.3390/v2091933.
9. Lelli, D.; Lavazza, A.; Prosperi, A.; Sozzi, E.; Faccin, F.; Baioni, L.; Trogu, T.; Cavallari, G.L.; Mauri, M.; Gibellini, A.M.; et al. Hypsugopoxvirus: A Novel Poxvirus Isolated from Hypsugo Savii in Italy. *Viruses* **2019**, *11*, 568, doi:10.3390/v11060568.
10. Erez, N.; Achdout, H.; Milrot, E.; Schwartz, Y.; Wiener-Well, Y.; Paran, N.; Politi, B.; Tamir, H.; Israely, T.; Weiss, S.; et al. Diagnosis of Imported Monkeypox, Israel, 2018. *Emerg. Infect. Dis.* **2019**, *25*, 980–982, doi:10.3201/eid2505.190076.
11. Vaughan, A.; Aarons, E.; Astbury, J.; Balasegaram, S.; Beadsworth, M.; Beck, C.R.; Chand, M.; O'Connor, C.; Dunning, J.; Ghebrehewet, S.; et al. Two Cases of Monkeypox Imported to the United Kingdom, September 2018. *Eurosurveillance* **2018**, *23*, 1800509, doi:10.2807/1560-7917.ES.2018.23.38.1800509.
12. Ng, O.T.; Lee, V.; Marimuthu, K.; Vasoo, S.; Chan, G.; Lin, R.T.P.; Leo, Y.S. A Case of Imported Monkeypox in Singapore. *Lancet Infect. Dis.* **2019**, *19*, 1166, doi:10.1016/S1473-3099(19)30537-7.
13. Nakazawa, Y.; Emerson, G.L.; Carroll, D.S.; Zhao, H.; Li, Y.; Reynolds, M.G.; Karem, K.L.; Olson, V.A.; Lash, R.R.; Davidson, W.B.; et al. Phylogenetic and Ecologic Perspectives of a Monkeypox Outbreak, Southern Sudan, 2005. *Emerg. Infect. Dis.* **2013**, *19*, 237–245, doi:10.3201/eid1902.121220.

14. Nakazawa, Y.; Mauldin, M.R.; Emerson, G.L.; Reynolds, M.G.; Lash, R.R.; Gao, J.; Zhao, H.; Li, Y.; Muyembe, J.-J.; Kingebeni, P.M.; et al. A Phylogeographic Investigation of African Monkeypox. *Viruses* **2015**, *7*, 2168–2184, doi:10.3390/v7042168.
15. Levine, R.S.; Peterson, A.T.; Yorita, K.L.; Carroll, D.S.; Damon, I.K.; Reynolds, M.G. Ecological Niche and Geographic Distribution of Human Monkeypox in Africa. *PLOS One* **2007**, *2*, e176, doi:10.1371/journal.pone.0000176.
16. Lash, R.R.; Carroll, D.S.; Hughes, C.M.; Nakazawa, Y.; Karem, K.; Damon, I.K.; Peterson, A.T. Effects of Georeferencing Effort on Mapping Monkeypox Case Distributions and Transmission Risk. *Int. J. Health Geogr.* **2012**, *11*, 23, doi:10.1186/1476-072X-11-23.
17. Likos, A.M.; Sammons, S.A.; Olson, V.A.; Frace, A.M.; Li, Y.; Olsen-Rasmussen, M.; Davidson, W.; Galloway, R.; Khristova, M.L.; Reynolds, M.G.; et al. A Tale of Two Clades: Monkeypox Viruses. *J. Gen. Virol.* **2005**, *86*, 2661–2672, doi:10.1099/vir.0.81215-0.
18. Berthet, N.; Descorps-Declère, S.; Besombes, C.; Curaudeau, M.; Nkili Meyong, A.A.; Selekon, B.; Labouba, I.; Gonofio, E.C.; Ouilibona, R.S.; Simo Tchetgna, H.D.; et al. Genomic History of Human Monkey Pox Infections in the Central African Republic between 2001 and 2018. *Sci. Rep.* **2021**, *11*, 13085, doi:10.1038/s41598-021-92315-8.
19. Curaudeau, M.; Besombes, C.; Nakoune, E.; Fontanet, A.; Gessain, A.; Hassanin, A. Identifying the Most Probable Mammal Reservoir Hosts for Monkeypox Virus Based on Ecological Niche Comparisons in review.
20. World Health Organization Smallpox: Post-Eradication Surveillance: First Isolation of Monkeypox Virus from a Wild Animal Infected in Nature. *Wkly. Epidemiol. Rec.* **1985**, *60*, 393–400.
21. Khodakevich, L.; Jezek, Z.; Kinzanzka, K. Isolation of Monkeypox Virus from Wild Squirrel Infected in Nature. *The Lancet* **1986**, *327*, 98–99, doi:10.1016/S0140-6736(86)90748-8.
22. Mariën, J.; Laudisoit, A.; Patrono, L.; Baelo, P.; van Vredendaal, R.; Musaba, P.; Gembu, G.; Mande, C.; Ngoy, S.; Mussaw, M.; et al. Monkeypox Viruses Circulate in Distantly-Related Small Mammal Species in the Democratic Republic of the Congo in review.
23. Tiee, M.S.; Harrigan, R.J.; Thomassen, H.A.; Smith, T.B. Ghosts of Infections Past: Using Archival Samples to Understand a Century of Monkeypox Virus Prevalence among Host Communities across Space and Time. *R. Soc. Open Sci.* **2018**, *5*, 171089, doi:10.1098/rsos.171089.
24. Kingdon, J.; Happold, D.; Butynski, T.; Hoffmann, M.; Happold, M.; Kalina, J. *Mammals of Africa*; Bloomsbury Publishing: London, 2013; ISBN 978-1-4081-2253-2.
25. Hebert, P.D.; Ratnasingham, S.; De Waard, J.R. Barcoding Animal Life: Cytochrome c Oxidase Subunit 1 Divergences among Closely Related Species. *Proc. R. Soc. Lond. B Biol. Sci.* **2003**, *270*, S96–S99, doi:10.1098/rsbl.2003.0025.
26. Petzold, A.; Hassanin, A. A Comparative Approach for Species Delimitation Based on Multiple Methods of Multi-Locus DNA Sequence Analysis: A Case Study of the Genus Giraffa (Mammalia, Cetartiodactyla). *PloS One* **2020**, *15*, e0217956, doi:10.1371/journal.pone.0217956.