Report 2024 of the PhD work of Manon Curadeau

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Funded Research Project :

A multidisciplinary approach to identify animal reservoirs of the monkeypox virus in Africa

« A One Health Study of Monkeypox: Human Infection, Animal Reservoir, Disease Ecology, and Diagnostic Tools »

Submitted by Institut Pasteur, Paris

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2024 Report

Summary:

The monkeypox virus (MPXV) was first identified in 1958 in laboratory macaques from Asia, but since then it has been understood the virus originates from Africa. Since then, MPXV has been detected in numerous African mammals, but their roles as reservoirs or secondary hosts remain unclear. In this study, **we aimed to identify the animal species that serve as reservoir hosts for MPXV.**

We first reconstructed MPXV's ecological niche using occurrence data from mammalian hosts and human index cases, showing that the niche spans the forests of Upper and Lower Guinea and the Congo Basin. We then compared this niche with those of 99 candidate mammals living in these forests. Our overlap analyses indicate that **the most likely reservoirs of MPXV are arboreal rodents in the forests of West and Central Africa, with Thomas's rope squirrel (*Funisciurus anerythrus*) as the top candidate.** Our model also suggests that this squirrel, previously recorded only in Central Africa, might also inhabit West Africa.

To test the hypothesis of coevolution between the virus and its host, we conducted a phylogeographic study of *F. anerythrus* by sequencing the genomes of 31 African squirrels, including 21 from the genus *Funisciurus*. Mitochondrial and nuclear analyses revealed that *F. anerythrus* is indeed present in West Africa. This group shows four geographic lineages corresponding to the Upper Guinea forest block, the relict forests of the Dahomey gap, the Lower Guinea forest block, and the Congo Basin. This geographic structuring aligns with MPXV's distribution, suggesting that its evolution has been strongly influenced by biogeographical barriers that limit or prevent the dispersal of these tree squirrels.

A multidisciplinary approach to identify animal reservoirs of the monkeypox virus in Africa

Work by Manon Curaudeau as part of her PhD thesis (nov 2021-nov 2024)

Mpox, previously referred to as monkeypox [1], is an emerging zoonotic infectious disease caused by the Monkeypox virus (MPXV), which is endemic to the rainforests of Central and West Africa. It affects individuals of all ages and typically progresses through three stages: incubation, prodromal, and eruptive. The prodromal stage is marked by symptoms such as fever, headache, fatigue, and often lymphadenopathy. In the eruptive phase, skin lesions appear in a centrifugal pattern, evolving through several stages: macules, papules, vesicles, and pustules. These lesions eventually form crusts that later desquamate [2, 3]

Mpox was first identified in 1958 at the *Statens Serum Institut* in Copenhagen, Denmark, in Asian macaques that were being used for polio vaccine production and research [4]. The first human case was recorded in 1970 in a young boy from Bokenda, in the Democratic Republic of the Congo (DRC) [5]. That same year, five additional cases were documented in West Africa: four in two villages in northeastern Liberia and one in southern Sierra Leone [6].

Since the 2000s, the number of reported cases has risen steadily, particularly in the DRC [7], from around a hundred cases a year in 2001 to several thousand in the late 2010s [8]. Sporadic cases have also been identified outside Africa in America, Asia, and Europe between 2003 and 2021 [9–16]. A global pandemic occurred in 2022, with over 100,000 cases reported, primarily affecting men who have sex with men [17, 18]. In response to the rapid spread of the virus, the World Health Organization (WHO) declared a global health emergency [19]. In 2023, another outbreak occurred in the DRC [20, 21], mainly linked to heterosexual human-to-human transmission, before spreading to neighbouring countries [22]. The WHO once again declared a global health emergency.

Although the reservoir of MPXV has not yet been definitively identified, several lines of evidence suggest that it is likely found among mammal species native to the rainforests of West and Central Africa. First, MPXV belongs to the genus *Orthopoxvirus* (OPXV), which exclusively infects mammals [23, 24]. Second, most human cases of mpox have been reported in the rainforests of Central and West Africa or in travellers returning from these regions [9–16]. Third, ecological niche modelling (ENM) has predicted that MPXV could potentially be found throughout all rainforests in these regions [11, 25–27]. Fourth, phylogenetic studies based on complete MPXV genomes have revealed a strong geographic structure [27–29]: viruses from Central Africa (Gabon, Cameroon, CAR, Republic of Congo, and DRC; clade I) are distinct from those in West Africa, which can be divided into two subgroups—one comprising viruses from Sierra Leone, Liberia, Côte d'Ivoire, and Ghana (clade IIa; [30]), and the other from Nigeria and Cameroon (clade IIb [30, 31]). This suggests that the animal reservoirs have been genetically isolated across three different rainforest regions for many generations: the Upper and Lower Guinean forests in West Africa and the Congo Basin in Central Africa. **Thus, we hypothesized that the MPXV reservoir is likely one or several mammal species whose geographic distribution closely mirrors that of MPXV.**

Identification of the most probable reservoir of MPXV with ecological niche modelling

In our first article [32], we investigated this hypothesis using a four-step approach: (i) we compiled a comprehensive list of mammal genera and species previously identified as natural hosts of MPXV in Africa; (ii) we predicted the geographic distributions (or ecological niches) of all species within these genera using georeferenced specimens from museum collection databases and ecological niche modelling (ENM) methods; (iii) we reconstructed MPXV's ecological niche by utilizing reliable data from human index cases and georeferenced MPXV sequences from wild animals; and (iv) we conducted statistical comparisons of the ecological niche overlaps between mammals and MPXV to identify the most likely mammalian reservoir(s).

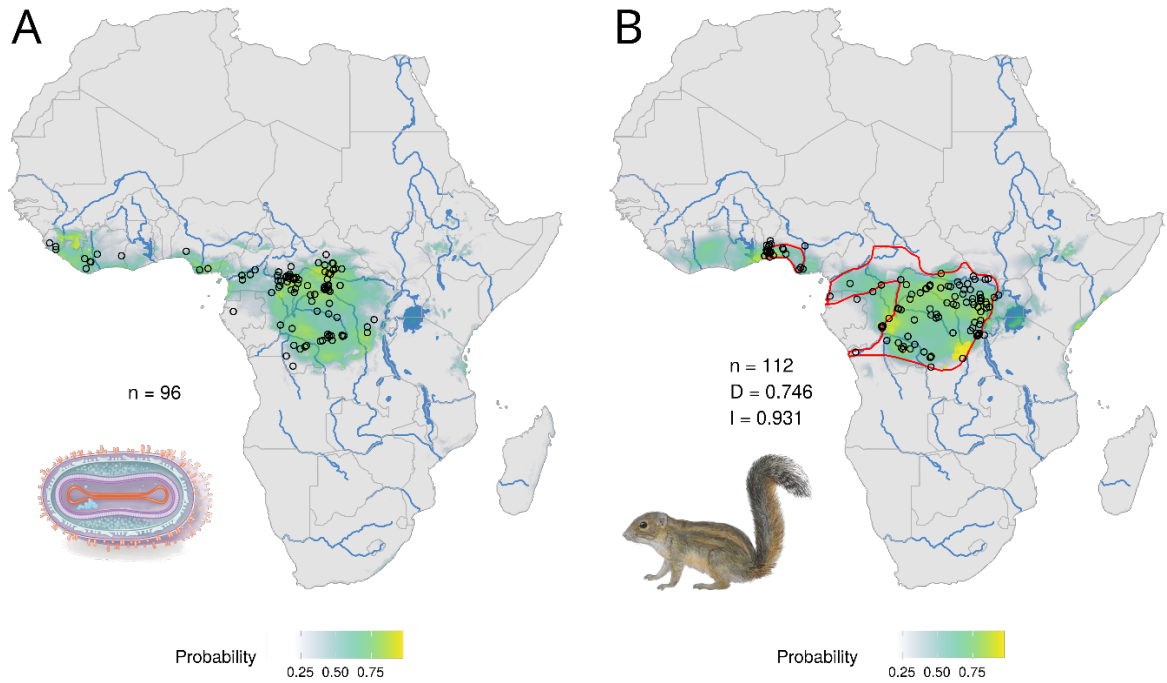

Figure 1. Ecological niches of Monkeypox virus (A) and *Funisciurus anerythrus*, the mammal species showing the best overlap with it (B). Black circles indicate localities used to build the distribution model. The probabilities of occurrence are highlighted using different colours: blue grey for probabilities < 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 (IUCN 2022). 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 *Funisciurus anerythrus* and MPXV. Adapted from Curaudeau et al. [32].

The results of our study confirmed that the ecological niche of MPXV spans three African rainforest regions: the Congo Basin and the Upper and Lower Guinean forests (Figures 1A). Our niche indicates that two biogeographic barriers have contributed to the isolation of mammalian populations hosting the MPXV reservoir. These barriers include the Volta River and Dahomey Gap, which separate the Upper Guinean forests (UGF) from the Lower Guinean forests (LGF) in West Africa, and the Sanaga River, which divides the LGF from the Congo Basin. **Our findings suggest that the most likely reservoir of MPXV is *Funisciurus***

***anerythrus*, an arboreal squirrel (Figure 1B).** Interestingly, this species is one of only two from which MPXV has been isolated and sequenced on two separate occasions by independent research teams [27, 33–35]

Phylogeographic studies of MPXV have demonstrated that this virus exhibits a strong geographic structure [27–29]. Based on the assumption that viruses co-evolve with their reservoir hosts, we would expect to observe similar phylogeographic patterns (i.e., the same geographic groupings) and comparable chronograms (estimations of divergence times). Since we have identified *Funisciurus anerythrus* as the most likely MPXV reservoir, we hypothesize that the phylogeography of this squirrel, based on DNA sequences from georeferenced individuals, should closely match that of MPXV. We therefore anticipate that the host phylogeny will show a deep division between Central Africa (corresponding to MPXV clade I) and West Africa (corresponding to MPXV clade II), along with similar subgroups within each major clade.

Furthermore, the data gathered on human index cases and wild animals enabled us to co-author two additional studies, published either during my year as an engineer or during my PhD. This data formed the basis for identifying important sequences in the first study, which explored the phylogeography of MPXV viruses [29]. The second study utilized this data to analyse the seasonal patterns of MPXV [36].

Phylogeographic comparison between MPXV and *F. anerythrus*

As a first step, we collected specimens of African Protoxerini squirrels, primarily from the genus *Funisciurus*, in collaboration with various museums and research institutions (including MNHN in Paris, RMCA in Tervuren, CBGP in Montpellier, AMNH in New York, FMNH in Chicago, MVZ in Berkeley, NMB in Basel, IVB in Brno, and ZFMK in Bonn). Total DNA was extracted from some of these samples, and a fragment of one mitochondrial gene was tested for amplification using polymerase chain reaction (PCR). We focused on the 5' region of the cytochrome c oxidase subunit I gene (COI; length = 705 bp), which serves as the barcode for species identification in molecular taxonomy [37]. Using COI sequences, we constructed a phylogenetic tree via the Maximum Likelihood method in IQ-TREE 2 [38], with 1,000 non-parametric bootstraps (Figure 2). **This tree represents the first phylogeny of African squirrels and revealed that several specimens in museum collections were likely misidentified, particularly *F. anerythrus* and *F. pyrropus*.**

We compared our phylogeny of georeferenced *Funisciurus* specimens to the phylogeography of MPXV previously published by our team [29]. Despite the limited squirrel dataset, **we observed a strong concordance between the two phylogeographies, suggesting a potential coevolution between MPXV and its likely reservoir host, *Funisciurus anerythrus*.** However, these initial findings are based on a small fragment of mitochondrial DNA, and previous phylogenetic studies have demonstrated that mitochondrial trees can sometimes be inconsistent with the species tree [39, 40]. This discordance may be due to differences in dispersal behaviors, as male mammals typically travel over greater distances while females tend to remain in their natal areas (female philopatry; [41, 42]. Since mitochondrial DNA is maternally inherited, a phylogeny based on mitochondrial sequences reflects only the

matrilineal history. Furthermore, interspecific hybrid females often remain fertile, unlike hybrid males (as per Haldane's rule), which can lead to mitochondrial introgression between closely related species [43].

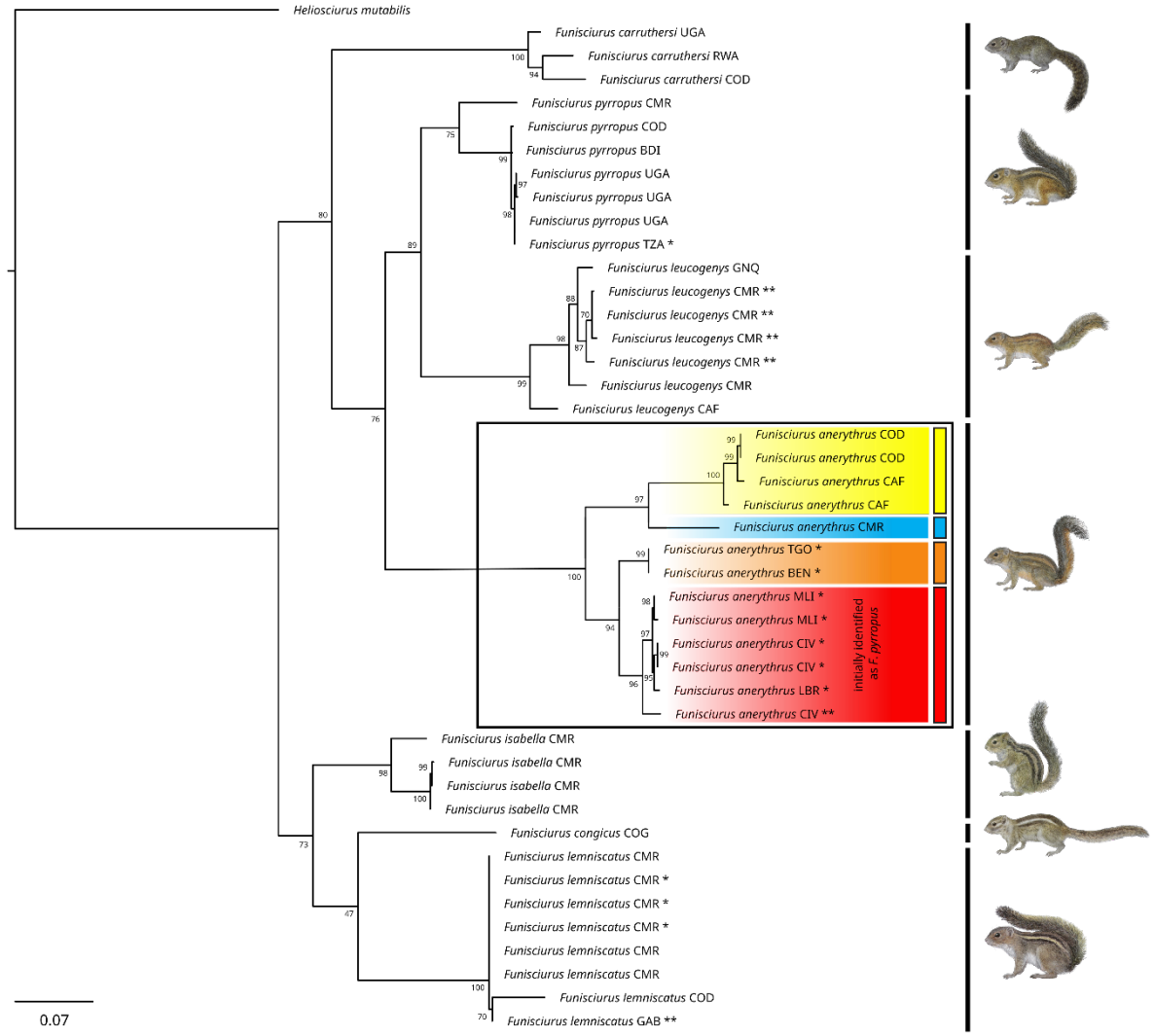

Figure 2. Phylogeny of *Funisciurus* species based on cytochrome c oxidase subunit I (COI) sequences. The phylogeny was reconstructed based on an alignment of COI sequences and the Maximum Likelihood method (software: IQ-TREE 2; 1,000 non-parametric bootstraps, bootstrap values indicated on the nodes). Some specimens were renamed in agreement with our phylogeny: those highlighted with a star were identified initially as *Funisciurus* sp.; those with two stars were identified initially as a different species. The specimens here identified as *Funisciurus anerythrus* are surrounded by a black box and geographic groups are coloured according to the MPXV geographic groups: red for the clade IIa, orange for the clade IIb, blue for the western-most subgroup of clade I, and yellow for the central subgroup of clade I.

Building on these initial findings, we chose to broaden our study by sequencing the complete genomes of various Funisciurus species rather than focusing solely on F. anerythrus. Specifically, we sequenced the entire genome for ten Funisciurus anerythrus specimens, eleven other Funisciurus species, and ten additional Protoxerini squirrels (Table 1).

Table 1: List of sequenced specimens

Institution	Species	Sex	Country
CBGP	*Funisciurus anerythrus*	Female	Benin
MNHN	*Funisciurus anerythrus*	Unknown	Cameroon
MNHN	*Funisciurus anerythrus*	Male	Central African Republic
MNHN	*Funisciurus anerythrus*	Female	Central African Republic
CBGP	*Funisciurus anerythrus*	Male	Côte d'Ivoire
MNHN	*Funisciurus anerythrus*	Unknown	Côte d'Ivoire
FMNH	*Funisciurus anerythrus*	Unknown	Democratic Republic of Congo
ZFMK	*Funisciurus anerythrus*	Female	Liberia
CBGP	*Funisciurus anerythrus*	Female	Mali
FMNH	*Funisciurus anerythrus*	Unknown	Togo
FMNH	*Funisciurus carruthersi*	Female	Democratic Republic of Congo
FMNH	*Funisciurus carruthersi*	Male	Rwanda
FMNH	*Funisciurus congicus*	Male	Democratic Republic of Congo
MNHN	*Funisciurus isabella*	Unknown	Cameroon
FMNH	*Funisciurus lemniscatus*	Female	Democratic Republic of Congo
FMNH	*Funisciurus lemniscatus*	Male	Gabon
MNHN	*Funisciurus leucogenys*	Male	Central African Republic
FMNH	*Funisciurus leucogenys*	Male	Equatorial Guinea
FMNH	*Funisciurus pyrropus*	Male	Burundi
MVZ	*Funisciurus pyrropus*	Female	Cameroon
MNHN	*Funisciurus pyrropus*	Male	Cameroon
CBGP	*Heliosciurus gambianus*	Male	Senegal
FMNHN	*Heliosciurus gambianus*	Male	Tanzania
FMNH	*Heliosciurus mutabilis*	Male	Mozambique
MNHN	*Heliosciurus rufobrachium*	Male	Cameroon
FMNH	*Paraxerus boehmi*	Male	Democratic Republic of Congo
MNHN	*Paraxerus cooperi*	Unknown	Cameroon
FMNH	*Paraxerus lucifer*	Male	Malawi
FMNH	*Paraxerus ochraceus*	Male	Tanzania
MNHN	*Paraxerus poensis*	Unknown	Cameroon
FMNH	*Protoxerus stangeri*	Male	Equatorial Guinea

Two datasets were created for the study. The first dataset comprised the complete mitochondrial genomes obtained using GetOrganelle v1.7.7.0 [44]. The second dataset included single-copy exons sourced from the Orthomam v10 database [45]. We extracted 167 exons, each over 1,000 bp in length, present in all specimens, with a GC content ranging from 40% to 60%. The phylogenetic trees were reconstructed with the Maximum Likelihood method using IQ-TREE 2 [38] with 1,000 non-parametric bootstraps. Divergence times were estimated on the concatenated alignment of all exons using the Bayesian approach implemented in BEAST v.2.7.7 [46].

The nuclear tree reveals a strong similarity to the mitochondrial tree (Figure 3). The sole topological differences concern the position of some outgroups (*Protoxerus* and *Heliosciurus*) and the monophyly of *Paraxerus*, as well as interrelationships between *Funisciurus anerythrus* from Côte d'Ivoire, Liberia, and Mali. **The comparative phylogeography of MPXV and *Funisciurus anerythrus* further confirms the presence of distinct geographic lineages (West Africa versus Central Africa; Mali + Côte d'Ivoire + Liberia versus Benin + Togo; Cameroon versus CAR + DRC), which bolsters the evidence for the crucial role of *Funisciurus anerythrus* as a reservoir host for MPXV.**

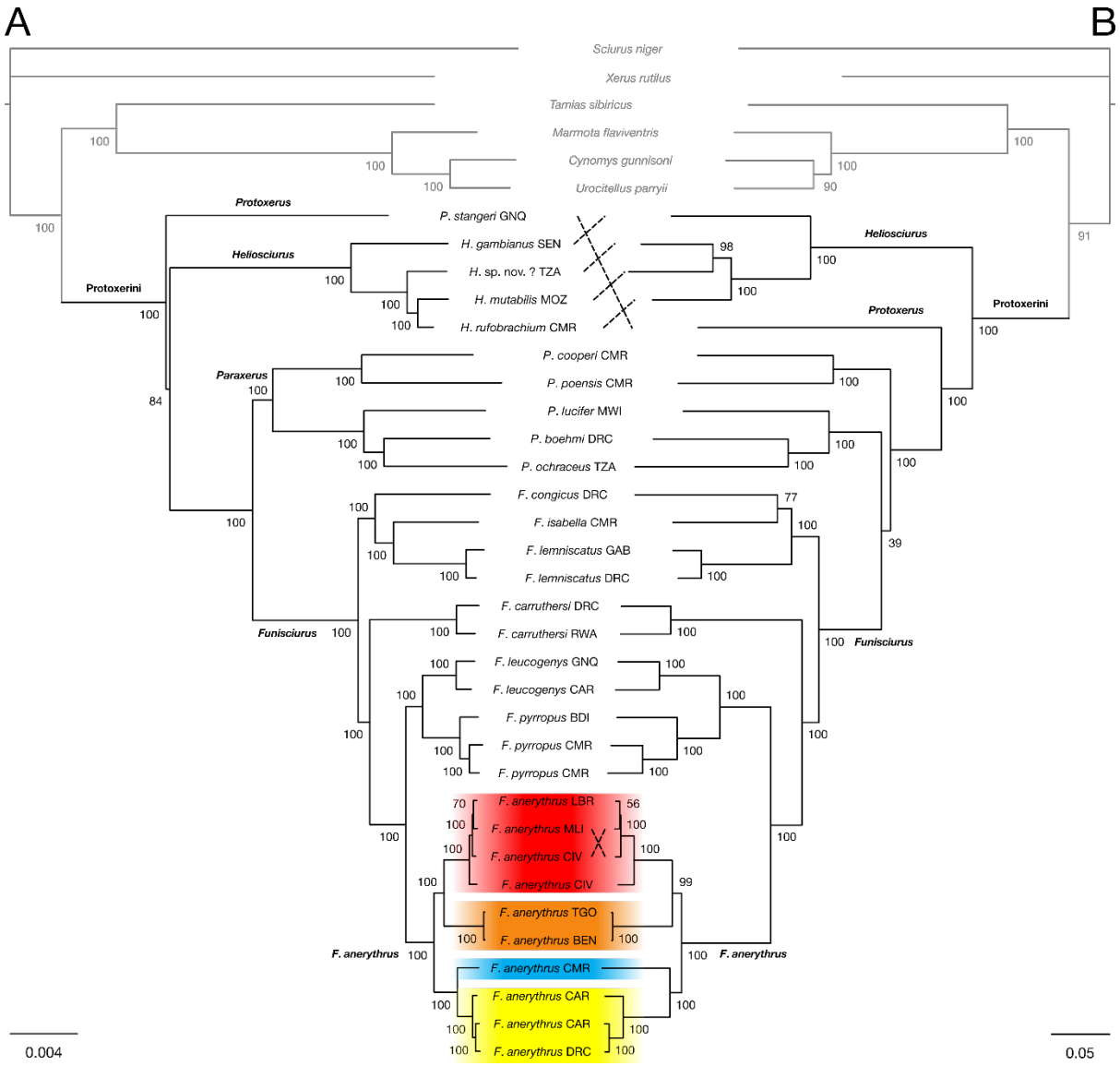

Figure 3. Phylogeny of Protoxerini species based on a concatenation of 167 nuclear exons (A) and the complete mitochondrial genome (B). The nuclear tree was reconstructed by the Maximum Likelihood method (software: IQ-TREE 2; 1,000 non-parametric bootstraps, bootstrap values indicated on the nodes) from an alignment of 37 taxa and 255,958 nucleotides. The mitochondrial tree was also reconstructed by the Maximum Likelihood from an alignment of 37 taxa and 16,326 nucleotides. Geographic groups of *Funisciurus anerythrus* are colored according to the MPXV geographic groups: red for the clade IIa, orange for the clade IIb, blue for the western-most subgroup of clade I, and yellow for the central subgroup of clade I.

Conclusion

An important and innovative feature of our project is that we will be using biological specimens (DNA) from major animal collections held in museums for years or decades. This **"museomics" work therefore avoids any field missions to collect animals in the forests of Central and West Africa.** There is therefore **no environmental impact, either in terms of carbon footprint, or on local animal biodiversity.**

Such studies, have two major goals.

The first one is to obtain better knowledge on the biodiversity and genetic aspects of the African squirrels and specifically those considered as the most probable reservoir for the MPXV.

The second one aims to provide to the public health actors and authorities of the different African countries, endemic/epidemic for Mpox, useful and informative data on such reservoir. This knowledge will favor the prevention of potential spillover from infected squirrel to humans and thus emergence of sporadic or epidemic Mpox.

Our publications on the project

Phylogeography of MPXV

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., Feher, M., Fontanet, A., Kazanji, M., Manuguerra, J.-C., **Hassanin, A.**, **Gessain, A.**, Nakoune, E., 2021. Genomic history of human monkey pox infections in the Central African Republic between 2001 and 2018. Sci Rep 11, 13085. https://doi.org/10.1038/s41598-021-92315-8

Identification of the probable reservoir of MPXV with ecological niche modelling

Curaudeau, M., Besombes, C., Nakouné, E., Fontanet, A., **Gessain, A.**, **Hassanin, A.**, 2023. Identifying the Most Probable Mammal Reservoir Hosts for Monkeypox Virus Based on Ecological Niche Comparisons. Viruses 15, 727. https://doi.org/10.3390/v15030727

Seasonnality of mpox cases

Besombes, C., Mbrenga, F., Gonofio, E., Malaka, C., Bationo, C.-S., Gaudart, J., **Curaudeau, M.**, **Hassanin, A.**, **Gessain, A.**, Duda, R., Giles Vernick, T., Fontanet, A., Nakouné, E., Landier, J., 2024. Seasonal Patterns of Mpox Index Cases, Africa, 1970–2021. Emerging Infectious Diseases 30, 1017. https://doi.org/10.3201/eid3005.230293

Phylogeographic comparisons between the MPXV and its reservoir

Curaudeau, M., Kerbis Peterhans, J., Demos, T., Goodman, S., Nicolas, V., Granjon, L., Denys, C., Missoup, A-D., Astrin, J., Nakouné, E., Fontanet, A., **Gessain, A.**, **Hassanin, A.**, *in preparation*. Comparative phylogeography of MPXV and it most probable reservoir (*Funisciurus anerythrus*).

Our presentations on the project :

In all presentations, the SCOR foundation logo appeared on the first and last slide, and it was mentioned the PhD was funded by the SCOR foundation. The SCOR foundation logo was also on the Poster.

Poster

In silico identification of Monkeypox virus reservoirs. **Curaudeau, M.**, **Gessain, A.**, **Hassanin, A.** Journées du département de Virologie de Pasteur, 16 May 2022, Le Pouliguen, France.

Presentations

Identification du réservoir mammifère le plus probable pour le virus Monkeypox à partir de comparaisons de niches écologiques. **Curaudeau, M.**, Besombes, C., Nakouné, E., Fontanet, A., **Gessain, A.**, **Hassanin, A.** Congrès des Jeunes Chercheur·e·s du Muséum, 5 May 2023, Paris, France.

Identification du réservoir mammifère le plus probable pour le virus Mpox à partir de comparaisons de niches écologiques. **Curaudeau, M.**, Besombes, C., Kerbis Peterhans, J. C., Nicolas, V., Granjon, L., Demos, T. C., Astrin, J., Goodman, S. M., Nakouné, E., Fontanet, A., **Gessain, A.**, **Hassanin, A.** Symposium international sur les maladies zoonotiques émergentes et réémergentes, 11 October 2023, Mbour, Senegal

Identification du réservoir mammifère le plus probable pour le virus Monkeypox. **Curaudeau, M.**, Kerbis Peterhans, J. C., Demos, T. C., Goodman, S. M., Nicolas, V., Granjon, L., Denys, C., Missoup, A-D., Astrin, J., Besombes, C., Nakouné, E., Fontanet, A., **Gessain, A.**, **Hassanin, A.** XXVIèmes Journées Francophones de Virologie, 12 avril 2024, Bruxelles, Belgium.

Identification du réservoir mammifère le plus probable pour le virus Monkeypox. **Curaudeau, M.**, Kerbis Peterhans, J. C., Demos, T. C., Goodman, S. M., Nicolas, V., Granjon, L., Denys, C., Missoup, A-D., Astrin, J., Besombes, C., Nakouné, E., Fontanet, A., **Gessain, A.**, **Hassanin, A.** Journées du département de Virologie de Pasteur, 14 mai 2024, Le Touquet, France.

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