# UCLA
## UCLA Electronic Theses and Dissertations

**Title**

An Investigation into the Historical Distribution, Prevalence, and Host Community of Monkeypox Virus (MPXV) Among Funisciurus Museum Skin Specimens from Central Africa

**Permalink**

https://escholarship.org/uc/item/47d295mf

**Author**

Tiee, Madeline

**Publication Date**

2015

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

An Investigation into the Historical Distribution, Prevalence, and Host Community of

Monkeypox Virus (MPXV) Among *Funisciurus* Museum Skin Specimens from Central Africa

A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in Biology

by

Madeline Shaoyun Tiee

2015

ABSTRACT OF THE THESIS

An Investigation into the Historical Distribution, Prevalence, and Host Community of

Monkeypox Virus (MPXV) Among *Funisciurus* Museum Skin Specimens from Central Africa

by

Madeline Shaoyun Tiee

Master of Science in Biology

University of California, Los Angeles, 2015

Professor Thomas Bates Smith, Chair

Monkeypox virus (MPXV), a zoonotic DNA virus, is now considered the most important

orthopoxvirus infection in human populations since the eradication of smallpox [1,2]. The role of

reservoir host species is highly important in this zoonotic disease due to the large percentage of

human infections attributed to wildlife origins [1,3]. As a disease that infects multiple hosts,

limited knowledge of MPXV prevalence across host species and the relative importance of each

host species to disease maintenance makes it difficult to predict human risk and target disease

management efforts. In this study, we screened over 1000 museum skin specimens collected

from 1899 to 1993 for viral DNA to understand historical MPXV infection in various

*Funisciurus* species across the Congo Basin. MPX viral DNA was found in 93 of 1038 (9.0%)

specimens from as early as 1899 and in five different *Funisciurus* species: *Funisciurus*

*anerythrus*, *Funisciurus carruthersi*, *Funisciurus congicus*, *Funisciurus lemniscatus*, and

*Funisciurus pyrropus*. We identify two new potential host species, gain insight into the relative

prevalence rates of infection within museum skins between various *Funisciurus* species, and

examine the spatial and temporal distribution of monkeypox virus in these possible host species.

The thesis of Madeline Shaoyun Tiee is approved.

James O. Lloyd-Smith

Anne W. Rimoin

Thomas Bates Smith, Committee Chair

University of California, Los Angeles

2015

TABLE OF CONTENTS

# LIST OF FIGURES AND TABLES

ACKNOWLEDGMENTS

**INTRODUCTION**:

Monkeypox virus (MPXV), a zoonotic DNA virus belonging to the genus *Orthopoxvirus*, is currently considered the most important human orthopoxvirus since the eradication of smallpox in 1977 [1,2]. In humans, MPXV causes clinical disease similar to smallpox including pustular rashes, fever, and lesions of mucous membranes but differs in its temporary lymphadenopathy [1,2,4,5]. MPXV is known to infect a broad range of hosts, including non-human primates, rodents, and humans. MPXV was first isolated from a colony of captive *Cynomolgus* monkeys in 1958, however reports of pox-like outbreaks as early as 1936 in non-human primates may be attributed to MPXV instead of smallpox [6]. In 1970, the first recognized human case was found in the Democratic Republic of Congo (DRC); and between 1970 and 1986, 404 human cases of MPXV infection appeared throughout Central Africa, predominately in the DRC but also within Sierra Leone, Liberia, and Cote d'Ivoire, Nigeria, Cameroon, and Central African Republic [2,5]. Since then, human populations in Central Africa have seen a dramatic 20-fold increase in MPXV infections in the past thirty years [7].

The transmission of MPXV between humans is often sporadic, clustered, and self-limiting, with multiple chains of transmission [1,8,9]. Previous studies suggest that a majority of human cases are primary or co-primary in nature, where the source of the infection is attributed to contact with wildlife. These studies, based on interview data from outbreaks in the 1980s, suggest primary cases comprise 72% [3] to as high as 78.1% [1] of all cases. If these estimates are accurate, a better understanding of host MPXV levels is crucial to estimating potential zoonotic spillover risk within human populations. While non-human primates and humans can be

1

incidental hosts of MPXV, rodents are considered the most likely candidates for reservoir hosts [1,5,10].

Our current knowledge of the role of MPXV reservoir species is limited; however the rodent families of *Funisciurus* (rope squirrels), *Heliosciurus* (sun squirrels), *Cricetomys* (Giant pouched rats), and *Graphiurus* (African dormice) have been implicated in Central and Western Africa for maintaining MPXV transmission [11–14,10,15]. Past surveys collectively found MPXV-specific antibodies in *Funisciurus anerythrus*, *F. congicus*, *F. isabella*, *F. lemniscatus*, *Heliosciurus gambianus*, *H. rufobrachium,* and *Cricetomys emini* [11–14,10]. In a study examining the 2003 importation of the West African strain of MPXV into the United States from Ghana, *Funisciurus*, *Cricetomys*, and *Graphiurus* were all found PCR-positive for MPXV DNA [16]. The only wild animal from which MPXV has been isolated was a singular moribund *F. anerythrus* in 1986 from Northern Zaire [12]. Multiple rodent species are infected with MPXV, however it is unknown which species are required to maintain MPXV within host communities. The natural history and ecology of arboreal squirrels belonging to the *Funisciurus* and *Heliosciurus* genera make them important candidates for spillover risk into human populations. These squirrels reach high densities, prefer secondary or degraded forest habitat such as agricultural lands close to human settlements, and are commonly hunted as bushmeat by children and young men [5,13,14].

The earliest serosurveys of MPXV within natural host populations occurred in 1979 [5]. As a DNA virus, MPXV offers the unique opportunity of using museum specimens to gain perspective into the historical prevalence and distribution of MPXV within host populations. Museum specimens have commonly been used to look at host genetic material, however recently

scientists have begun to use archival samples to understand pathogens [17,18]. For pathogens, which often occur at lower prevalence levels, museum collections offer samples that span a wide range of years, locations, and species without the effort of intensive field sampling [17]. While museum specimens come with their own sources of error, they can be valuable for understanding broad geographical patterns of disease, especially in areas that are difficult to sample. This type of study using museum specimens has not been done on MPXV in host species, however orthopoxvirus DNA has been recovered successfully from human corpses exhumed from permafrost and historical human scab specimens [19]. Here we present our results screening 1038 *Funisciurus* museum specimens from Central Africa for MPX viral DNA. Our samples span 1899 to 1993 and are predominately from the DRC, but also include another surrounding 12 countries in Central and West Africa. Species sampled include *F. anerythrus*, *bayoni*, *carruthersi*, *congicus*, *isabella*, *lemniscatus*, *leonis*, *leucogenys*, *pyrropus*, and *substriatus*. The objectives of our study were to: (i) investigate the potential for using museum skin specimens in surveying the prevalence of MPXV, (ii) compare the prevalence levels of MPXV within various purported host species, (iii) examine the spatial and temporal patterns of MPXV prevalence, and (iv) explore the impact of particular host communities and richness on MPXV prevalence.


**MATERIALS & METHODS:**

*Sample collection:*

*Funisciurus* skin samples were collected from specimens housed at two different museums: Royal Museum for Central Africa (RMCA) in Tervuren, Belgium and the American Natural History Museum (AMNH) in New York City, New York, USA. Samples from the RMCA were collected August 20-25, 2012 and samples from the AMNH were collected September 8-9 and

October 14, 2014. Permission for sampling at both museums was granted by mammal collection curators prior to sample collection.

A 9 to 25 mm-squared sample was collected from the ventral side of the proximal axillary front legs (armpit), ventral side of lower neck, or ventral midline with an effort to minimize visible damage to specimens. Samples were kept dried at ambient temperature in 1.7-mL tubes prior to DNA extraction. Past laboratory experiments on the 2003 USA outbreak of MPXV within rodent populations showed that skin served as a reliable tissue for MPXV DNA detection [16].

In all, 1077 *Funisciurus* skin samples were collected: 377 *F. anerythrus*, 7 *bayoni*, 112 *carruthersi*, 252 *congicus*, 18 *isabella*, 84 *lemniscatus*, 2 *leonis*, 6 *leucogenys*, 207 *pyrropus*, 1 *substriatus*, and 11 *Funisciurus* specimens not identified to species level. A total of 748 samples were from the RMCA, and 329 were from the AMNH.

***DNA Extraction and Quality Assessment***

DNA extraction was performed using a Qiagen DNeasy Blood and Tissue Kit (Hilden, Germany) according to the manufacturer's recommendations. DNA concentration and purity were assessed through the use of a Thermo Fisher Scientific NanoDrop 2000 full-spectrum UV-Vis spectrophotometer (Waltham, Massachusetts, USA). DNA extracts were stored at -20°C.

Overall DNA quality was assessed through PCR amplification of the common vertebrate gene beta-actin. The forward primer for the beta-actin amplicon was 5'– CCCTGAAGTACCCCATTGAA–3' and the reverse primer was 5'–

CTTGAAGGTCTCAAACATGATCT–3' (Integrated DNA Technologies Inc., Coralville, Iowa, USA). This 188-bp amplicon was similar in length to the two amplicons used to screen samples for MPXV viral DNA and served as a valid proxy of DNA quality and degradation. All beta-actin PCR reactions were run using the Qiagen Multiplex PCR Kit (Hilden, Germany) and contained 1.5 μL of template DNA, 1 μL of 1 μM primer for both forward and reverse primers, 5 μL of 2x Qiagen Multiplex PCR Master Mix, 1 μL 5x Q-Solution, 0.4 μL of 10 mg/mL bovine serum albumin, and 1.1 μL of RNase-free water. PCR reactions were run on 96-well plates with one negative using 1.5 μL Qiagen AE Elution buffer (Hilden, Germany) in place of template DNA. The reaction was run on a MJ Research PTC-200 Peltier Thermal Cycler (MJ Research Inc., Waltham, Massachusetts, USA) with thermal cycling conditions including initial denaturation for 15 mins at 95°C, followed by 11 cycles of 30 s denaturation at 94°C, 90 s annealing phase at 60°C, and 60 s extension at 72°C, then 31 cycles of 30 s denaturation at 94°C, 90 s annealing at 55°C, and 60 s extension at 72°C, followed by a final extension for 30 mins at 60°C. All PCR products were stored at -20°C.

PCR products were run on a 2% agarose gel and visualized using SYBR Safe DNA gel stain (Invitrogen, Eugene, Oregon, USA). PCR reactions were repeated up to four times, and samples in which beta-actin could not be amplified were dropped from the study to avoid false-negatives. In all, 39 samples from the 1077 total were excluded from the final data due to lack of beta-actin amplification. The final number of samples screened for MPXV DNA was 1038: 362 *F. anerythrus*, 7 *bayoni*, 109 *carruthersi*, 239 *congicus*, 18 *isabella*, 82 *lemniscatus*, 2 *leonis*, 6 *leucogenys*, 201 *pyrropus*, 1 *substriatus*, and 11 *Funisciurus* specimens not identified to species level. A total of 92 samples were from Angola, 3 from Burundi, 26 from Cameroon, 8 from the

Central African Republic, 69 from the Republic of Congo, 747 from the Democratic Republic of

Congo (DRC), 2 from Equatorial Guinea, 15 from Gabon, 3 from Ghana, 13 from Cote d'Ivoire,

2 from Liberia, 13 from Rwanda, 1 from Uganda, and 44 with unknown locality.


Additionally, some samples were analyzed using the Agilent 2100 Bioanalyzer and the Agilent

High Sensitivity DNA kit (Agilent Technologies, Santa Clara, California, USA) to look at DNA

quality and fragment length and to ensure that samples were suitable for MPXV screening

attempts. From the analysis of 8 random samples, the average length of DNA in our samples was

218-bp (range=29 –1000 bp).


*MPXV Screening*

Samples were screened for MPX viral DNA using two different amplicons G2R_G and

G2R_WA developed by Li *et al.* [20]. Both amplicons are from the conservative tumor necrosis

factor (TNF) receptor gene located within the inverted terminal repeat (ITR) region. G2R_G

contains one single nucleotide polymorphism and G2R_WA includes a 3-bp deletion in the

Congo-Basin strain to allow the distinction between the West African and the Congo Basin

strains. For the G2R_G amplicon, we used the same reverse primer designed by Li *et al.* [20]:

5'–GCTATCACATAATCTGGAAGCGTA–3'. Primer3 version 4.0.0 [21,22] was used to create

a forward primer for G2R_G:  5'–GGAAGAGATATAGCACCACATGC–3'. For the G2R_WA

amplicon, we used a forward primer that was almost identical to that used in Li *et al.* [20] but

was shifted by 1-bp for a forward primer of 5'–TCACACCGTCTCTTCCACAG–3'. Primer3

[21,22] was used to create the optimal reverse primer for G2R_WA: 5'–

ACGATGTGTCGTTGACTGGA–3' (Integrated DNA Technologies Inc., Coralville, Iowa, USA

and ValueGene Inc., San Diego, California, USA). G2R_G is a 123-bp amplicon, and G2R_WA is a 101-bp amplicon with the Congo Basin Strain deletion or a 104-bp amplicon for the West African strain. All real-time PCR reactions contained 1 μL of template DNA, 0.50 μL of 1 μM concentration of each primer, 5 μL of the Roche LightCycler 480 High Resolution Melting Master Mix 2x concentrated, 1.75 μL of 25 mM magnesium (II) chloride, and 2.25 μL of de-ionized water (Roche Diagnostics, Basel, Switzerland).

Samples were loaded onto a clear 384-well plate in four replicates for each amplicon. Amplicons were run on separate plates. Four negative controls using 1 μL of Qiagen Elution AE buffer (Hilden, Germany) in place of template DNA were run on each plate. For the first amplicon G2R_G, no positive controls were used to prevent contamination. For the second amplicon, an altered positive control oligo was used (Integrated DNA Technologies Inc., Coralville, Iowa, USA). This positive control oligo had a 5-bp section in the middle altered in sequence to allow discrimination between positive control contamination and wild positives. Six serial dilutions of the positive control were placed on each plate for the G2R_WA amplicon at concentrations of 0.1nM, 0.01 nM, 1 pM, 0.1 pM, 0.01 pM, and 0.001 pM.

MPXV viral DNA was amplified using real-time PCR (RT-PCR) assays on a Roche LightCycler 480 Real-Time PCR System (Roche Diagnostics, Basel, Switzerland). A touchdown PCR protocol with thermal cycling conditions of an initial denaturation cycle of 10 min at 95°C followed by 60 cycles of 10 s denaturation at 95°C, 15 s annealing at 65 to 53°C, and 10 s extension at 72°C. The annealing temperature began at 65°C and decreased with each cycle by 0.5°C until reaching a final annealing temperature of 53°C which was held for the remainder of

cycles. After the completion of PCR, high-resolution melting (HRM) analysis was performed on all samples to confirm PCR specificity; the high-resolution melting cycle included 60 s at 95°C, 60 s at 40°C, 1 s at 65°C, and then heating to 97°C with a ramp rate of 0.02°C/s. The combination of RT-PCR and HRM analysis allows for the amplication and sensitive detection of DNA at low copy numbers [23].

Melt temperatures ($T_m$) of RT-PCR products were determined through analysis of melt curves using Roche LightCycler 480 Software-version 1.5.039 (Roche Diagnostics, Basel, Switzerland). Putative MPXV positives were detected through examination of the $T_m$. All positives of the G2R_G amplicon melted on average at 79.6°C (95% CI, 79.37 – 79.83°C), and all G2R_WA amplicons melted at an average 78.89 °C (95% CI, 78.66 – 79.13°C). Any $T_m$ peaks at temperatures outside of the melting temperature were attributed to non-specific amplification or primer-dimer. Due to the low copy number of pathogen DNA in comparison to host DNA, HRM ensured high sensitivity to viral DNA amplification. All RT-PCR products were stored at -20°C.

All HRM putative positives were sent to Beckman Coulter Genomics (Danvers, Massachusetts, USA) for product purification and Sanger DNA sequencing. Sequences were aligned to the G2R_G and G2R_WA amplicons using Geneious version 7.1.3 [24]. Samples were considered positive for MPXV DNA if they could be sequenced for either the G2R_G or G2R_WA amplicons.

*Authenticity of Positives*

To prevent contamination, Cooper and Poinar [25] have recommended nine criteria for the authenticity of museum studies. As suggested by Gilbert *et al.* [26], we have designed our study to avoid contamination but have not used all recommended "nine criteria of authenticity" because our study system falls into a low-risk category. The low-risk category includes studies that do not investigate ancient human DNA, paleopathogens and microorganisms for which modern counterparts are ubiquitous, or domestic plants/animals [26]. As MPXV is a non-ubiquitous pathogen, we implemented five of the nine criteria: (1) physically isolated work areas, (2) negative controls, (3) appropriate molecular behavior, (4) quantification by RT-PCR, and (5) associated remains. All PCR products and positive controls were stored in a room and freezer separate from where DNA extractions and RT-PCR plate preparation occurred. All plates were run with four negative controls: no negative controls came up positive during the course of the study. All positive controls used modified sequences to allow differentiation of positive control contamination from actual positives. Additionally, the use of Sanger sequencing as a final verification step ensured that any wild contamination that happened at low concentrations would sequence poorly. A comparison of putative HRM positives to Sanger-sequence verified positives shows that HRM was far more sensitive to DNA amplification at low copy numbers than Sanger sequencing (Fig S1). Furthermore, we amplified viral DNA of short length (<500 bp) and expected our shorter amplicon G2R_WA to amplify more often than our longer amplicon G2R_G [25,27]. Additionally, we were able to quantify all our reactions using RT-PCR. All reactions amplified late in the cycles, however this is normal for museum studies, especially involving the amplification of something in low copy number like that of viral DNA within more

concentrated host DNA [18]. Our "associated remains" included using host DNA quality and amplification of the common gene beta-actin as a proxy for MPXV viral DNA quality [18].

***Statistical Methods***

*Comparing MPXV infection across species, museum, and year of collection*

All statistical analyses were done using RStudio version 0.98.1091 [28] and R version 3.0.2 [29]. Fisher exact tests of independence were performed and mixed effects binomial logistical regression models were fit to data to compare MPXV positive outcome across species, collection years, collection months, museum, age of sample, DNA quality (NanoDrop 260/280 ratio), DNA concentration (ng/µL), sex, and age of specimen (juvenile vs. adult) using R packages stats and car [29,30]. Tukey's all pairwise difference tests were conducted to compare MPXV outcome between species and years using R package multcomp [31]. Collection year was binned by five year intervals to increase sample sizes across years except for the interval 1899-1905: 1906-1910, 1911-1915, 1916-1920, etc. Model significance was examined using likelihood-ratio chi-squared tests.

*Comparing host community composition to MPXV infection*

To understand the effects of host community composition on MPXV infection, MPXV positive counts were analyzed across administrative areas and compared to the presence and absence of various possible host species. Most museum specimen samples were geo-referenced to town level, however some were geo-referenced to the sous-préfecture, district, province, or country level. All localities for museum specimens were verified or found using the National Geospatial-Intelligence Agency's GEOnet Names Server database [32] and Google Earth Landsat (Google,

Landsat, SIO, NOAA, US Navy, NGA, GEBCO 2015). Due to differences in localities' spatial extents, samples were analyzed at a lower spatial resolution and aggregated by administrative area. These administrative areas differed in level between countries but were chosen based on similarity in size: sous-préfecture (DRC), préfecture (Rwanda), município (Angola), district (Ghana and Republic of Congo), département (Cameroon, Cote d'Ivoire, and Gabon), province (Burundi and Equatorial Guinea), and county (Liberia) levels. All samples with spatial extents greater than these administrative areas were dropped from this analysis.

Potential host distributions were obtained from the IUCN *Red List of Threatened Species Version 2012.1* [33] for genera *Funisciurus* and *Heliosciurus*: *F. anerythrus*, *F. bayoni*, *F. carruthersi*, *F. congicus*, *F. isabella*, *F. lemniscatus*, *F. leonis*, *F. leucogenys*, *F. pyrropus*, and *H. gambianus*, *H. rufobrachium*, and *H. ruwenzorii*. Presence/absence of a species was coded as a binary variable at a 1-km spatial resolution and averaged across administrative areas. Additionally, various combinations of host community richness were calculated. Other indices of richness included the species richness of (i) genus *Funisciurus*, (ii) genus *Heliosciurus*, (iii) all species belonging to both genera, (iv) the presence of both *F. anerythrus* and *F. congicus*, (v) host species found to be MPXV positive in this study (*F. anerythrus*, *carruthersi*, *congicus*, *lemniscatus*, and *pyrropus*), and (vi) host richness found seropositive historically and PCR-positive in this study (*F. anerythrus*, *carruthersi*, *congicus*, *isabella*, *lemniscatus*, *pyrropus*, *gambianus*, *rufobrachium*). All spatial resampling, extraction of raster values, creation of species richness indices were performed using a combination of QGIS Version 2.6.0-Brighton [34], RStudio version 0.98.1091 [28], R version 3.0.2 [29], R package raster version 2.3-12 [35], and R package rgdal version 0.9-1 [36].

MPXV positive counts per administrative area were compared to the presence/absence of specific species and other indices of species richness using a mixed effects Poisson regression model with a logit link. Models were created for each MPXV-positive species separately and controlled for variation between years and sampling effort by including binned years as a random effect and an exposure variable. The exposure variable offset the model by a logarithmic function of the total number of samples from each administrative area, adjusting the model to account for differences in opportunity to detect an MPXV positive due to sample size. The same analysis was repeated with the addition of museum as a random effect to see the effect that the sample provider had on these models.

All mixed effects models were fit to the data using the R package lme4 version 1.1-7 [29,37]. Model selection was done through R package MuMIn version 1.13.4, which ranks all possible model permutations by AIC value and weights their importance in the overall model [29,38]. Individual model variables were tested for significance using chi-squared tests comparing models with and without each variable in the model, and overall model fit was measured through pseudo R-squared values and F-test. The residual deviance was compared to residual degrees of freedom to ensure that the data did not exhibit issues of overdispersion.

**RESULTS:**

The overall MPXV prevalence level of samples found positive for either MPXV amplicon was 9.0% (93/1038). All samples, as revealed by their sequences, were of the Congo Basin strain of MPXV. All positive samples, except for one in the Central African Republic, were found in the

DRC with large numbers of positives occuring in the northwestern provinces of Kivu and

Orientale, the southern province of Kasai-Occidental, and the eastern provinces of Bandundu,

Équateur, and Bas-Congo (Fig 1, Fig. S2).

### *Differences in MPXV Prevalence by Species*

Of the ten species tested, five species had at least one individual positive for MPXV DNA:

*Funisciurus anerythrus, carruthersi, congicus, lemniscatus,* and *pyrropus*. MPXV DNA for

either amplicon was amplified in 12% (45/362) of *anerythrus* specimens, 2.8% (3/109) of

*carruthersi*, 13% (32/239) of *congicus*, 6.1% (5/82) of *lemniscatus*, and 4.0% (8/201) of

*pyrropus* (Fig 2, Fig S3, Table 1). All other species did not screen positive for MPXV DNA: *F.*

*bayoni* (0/7), *isabella* (0/18), *leonis* (0/2), *leucogenys* (0/6), *substriatus* (0/1), and *F. spp.* (0/11).

When broken down by amplicon, the prevalence rates for MPXV DNA using only the G2R_G

amplicon was overall 3.4% (35/1038), 3.3% (12/362) for *F. anerythrus*, 0.92% positive (1/109)

for *F. carruthersi*, 6.3% positive (15/239) for *F. congicus*, 2.4% positive (2/82) for *F.*

*lemniscatus,* and 2.5% positive (5/201) for *F. pyrropus*. For only the G2R_WA amplicon, the

overall MPXV prevalence rate was 6.9% (72/1038), 10% (38/362) for *F. anerythrus*, 1.8%

positive (2/109) for *F. carruthersi*, 10% positive (24/239) for *F. congicus* 3.7% positive (3/82)

for *F. lemniscatus*, and 2.5% positive (5/201) for *F. pyrropus* (Fig S4, Table 1).

Based on a Pearson's Chi-squared test of independence, the null hypothesis that different species

have equal mean prevalence was rejected ($p = 0.0021$, $\chi^2 = 27.61$, $df$=10). Using a mixed effects

binomial logistic regression model fit to the MPXV positive/negative outcome data with species

as the independent variable and year bin and museum as random effects, the overall likelihood-ratio chi-squared test showed species is a significant factor in predicting MPXV positive outcome ($p = 0.00082$, $LR\ \chi^2 = 30.106$, $df = 10$). In comparison to *F. anerythrus*, a negative coefficient for *carruthersi* ($\beta = -1.58$), *lemniscatus* ($\beta = -0.18$), and *pyrropus* ($\beta = -1.10$) show that these three species are less likely to be positive for MPXV. *F. congicus* however had a positive coefficient ($\beta = 0.50$), suggesting that a *congicus* sample has slightly higher odds of being positive when compared to *anerythrus*. Tukey's all pairwise differences comparing the mean MPXV prevalence of various species to each other found significant differences between the means of *congicus* and *carruthersi* ($p=0.023$) and *congicus* and *pyrropus* ($p=0.0069$). Fisher exact tests revealed no significance of sex ($p=0.21$) or age of host ($p=0.61$) as a factor in MPXV positive outcome.

***Temporal Differences in MPXV Prevalence***

MPXV positive samples spanned 94 years (1899 to 1993), and there were some general differences seen between year bins. A mixed effects binomial logistic regression model fit to MPXV positive/negative outcome data with binned years as the independent variable and species and museum as random effects was significant ($p=0.0026$, $LR\ \chi^2 =36.28$, $df = 16$). Tukey's all pairwise differences comparing the means of various year bins found a significant difference after adjusting for multiple comparisons in the means between year bins 1956-1960 and 1921-1925 (*Multiplicity adjusted p* $= 0.028$), and slight differences between year bins 1956-1960 and 1911-1915 (*Adj. p* $= 0.087$) and between 1956-1960 and 1931-1935 (*Adj. p* $= 0.054$). When samples were binned by other year intervals, there still appeared to be similar trends with a general period of higher MPVX prevalence between 1911 and 1935 and a lower MPXV

14

prevalence in the late 1950s (Fig. S5-S6). For example using samples binned by about 10-year intervals, Tukey's pairwise differences of MPXV outcome was significant between the year bins of 1908-1918 and 1950-1960 and between 1919-1928 and 1950-1960. No significant trends were seen by month. (Fig S7). No significant temporal trends were seen across years within administrative areas due to low sampling in the same administrative area across multiple years.

To understand possible effects of DNA quality on MPXV outcome, a mixed effects binomial logistic regression model with DNA quality (260/280 Nanodrop ratio), DNA concentration, and age of sample (year) as independent variables and museum as a random effect were fit to MPXV outcome. Similar to the temporal dynamics seen in collection year, the age of sample was the only significant variable. When a mixed effects logistic regression was fit to MPXV outcome with age of sample as the only independent variable and museum as a random effect, the model overall is significant ($p$=1.22E-6, $LR\ \chi^2$ =23.54, $df$ = 1), and the coefficient of this sample age variable is small and positive ($\beta_{sample\ age}$=0.029), meaning that as the age of the sample increased, the likelihood of having a MPXV-positive outcome increased slightly. Our DNA does not seem to be any more degraded in older or MPXV-negative specimens, which suggests that our prevalence levels likely reflect true relative differences between sampling periods.

***Differences in MPXV Prevalence by Museum Collection***

A significant difference in MPXV outcome was seen in specimens based on the museum that the specimen was from. The geographic distribution of samples varied widely by museum and much of the overlap occurred in the DRC. When the data is constrained to the administrative areas where museums overlap, a mixed effects Poisson regression model fit to MPXV counts with year

bin as a random effect and museum as a variable was significant in *F. anerythrus* (*p*=0.0015, *LR*

$\chi^2$ =10.05, *df* = 1) and *F. congicus* (*p*=0.046, *LR* $\chi^2$ =3.97, *df* = 1). Both saw a higher likelihood

of MPXV positives for the RMCA specimens. Additionally, the concentration of DNA extracted

from specimens was correlated to the museum origin. In a significant linear regression model

fitted to the concentration of DNA (*p*=0.017, *F*=5.68, *df* = 1, 1001), DNA concentration of the

RMCA was 13.66 ng/μL higher than that of the AMNH specimens. Likewise, a linear model

examining the relationship of DNA quality (260/280 ratio from the Nanodrop) showed a slightly

significant (*p*=0.055, *F*=3.69, *df* = 1, 1001) association with slightly higher quality in the RMCA

samples (*β* = 0.042).


***Host Community Effects:***

Poisson models for species *F. anerythrus*, *carruthersi*, *lemniscatus*, and *pyrropus* did not reveal

any significant factors in determining MPXV positive count. For *congicus*, 10 of the 11 top

models when ranked by AIC value included the presence/absence of *anerythrus.* Additionally,

the top ranked model included the presence/absence of *anerythrus* as its only independent fixed

variable. In this top model, *congicus* positive MPXV counts were fitted to a Poisson mixed

effects model where year bin was held as a random effect and the presence/absence of

*anerythrus* was the fixed effect. The model was significant (*R$^2$*=0.4306, *p*=7.6E-5, *F-value* =16,

*df* = 1, 57) and the fixed variable of *anerythrus* presence/absence was significant in the model

(*p*=3.41E-6, *LR* $\chi^2$ =21.57, *df* = 1). The positive coefficient of 2.91 (*SE*= 0.86, *z-value* = 3.93,

*p*=0.00069) for the *anerythrus* presence/absence variable suggests that the presence of

*anerythrus* in the same administration area results in an 18.41 fold increase of MPXV positives

in *congicus*. The reverse effect of the presence/absence *congicus* on *anerythrus* was not seen; the

model using *congicus* co-occurrence as the explanatory variable did not significantly predict *anerythrus* MPXV positive counts ($p$=0.93, *LR* $\chi^2$ =0.0086, *df* = 1). This may underscore the importance of *anerythrus* within interspecific transmission in MPXV dynamics or it may also be indicative of underlying environmental or host constraints that may limit what host-environment is suitable for MPXV survival to the range of *anerythrus*.

However, a large caveat to this result is that all the MPXV-negative *congicus* specimens in Angola where *congicus* and *anerythrus* do not co-occur are from the AMNH museum where MPXV positives were generally much lower. When museum was added as a random effect in the mixed effects Poisson regression models, there were no significant patterns seen in host community composition for any of the species.

**DISCUSSION:**

This is the first study to survey MPXV within host species using museum specimens, and our findings suggest there is great potential for using museum collections to retrospectively investigate the historical prevalence and distribution of DNA viruses. In our study, we have expanded on the knowledge of MPXV infection within host species over the past century within the genus *Funisciurus*. We found evidence of MPXV circulating in host species as early as 1899, over 115 years ago, supporting the suggestion that pox-like outbreaks in humans and non-human primates could have historically been caused by MPXV instead of smallpox [4,6]. Additionally, we have identified two new potential host species *F. carruthersi* and *pyrropus* and have verified MPXV infection within the host species of *F. anerythrus*, *congicus*, and *lemniscatus*. Until this study, all evidence of MPXV infection of the Congo-Basin strain in wild rodent populations,

except for the singular isolation of MPXV virus in *F. anerythrus,* has historically been serological in nature [5,11–13]. Among the species found to harbor MPX infections, there appear to be striking differences in MPXV prevalence. *F. anerythrus* and *congicus* both had high MPXV prevalence levels within the collected museum skin specimens. These two species also have the largest distributional ranges in the DRC, suggesting that they may play more major roles in the transmission of MPXV within host communities and in spillover into humans (Fig S2).

We also found general temporal differences in MPXV prevalence, with higher prevalence levels between 1911 and 1935 as compared to the late 1950s. This difference in MPXV prevalence could be a true signal of differences in MPXV between years or may be attributed to sampling bias and environmental spatial heterogeneity. The spatial distribution of our samples was not even across years, meaning that the areas sampled from 1911 to 1935 do not overlap perfectly with the areas sampled in the late 1950s. This suggests the possibility that there is spatial heterogeneity in MPXV prevalence across the DRC and that sampling in earlier years may have included areas of general higher MPXV prevalence by chance while sampling in later years occurred in areas with less MPXV. One possibility for this spatial heterogeneity is the distribution of ideal squirrel habitat; rope squirrels generally inhabit humid lowland evergreen tropical forests but prefer degraded habitat, agricultural lands, and palm oil plantations [13,14,39]. Palm oil trees (*Elaeis guineensis*) in particular provide *Funisciurus* populations with a steady food source, allowing squirrels to reach higher densities in these degraded forests with oil palm trees, ranging anywhere from 440 to 500 squirrels per square kilometer [13,14,39].

Of interest is what factors limit the distribution of MPXV in *F. congicus* to the areas also inhabited by *F. anerythrus* in the DRC. Generally all positives identified were within this similar geographic area. This could be due to several different reasons: (1) *F. anerythrus* is the primary reservoir host and allows for infection in species that co-occur with it, however other hosts may not be able to sustain transmission alone, (2) the community of *anerythrus* and other *Funisciurus* species that co-occur maintain the virus together by forming various maintenance communities across space, (3) there are other environmental constraints that limit MPXV to the same area occupied by *F. anerythrus*, (4) low sampling and specimen collection across the entire range has missed positive locations, and (5) differences between museums in specimen preparation/storage affect the ability to detect viral DNA. Khodakevich *et al.* [13] have suggested that *F. anerythrus* is the main host sustaining transmission of MPXV in Central Africa, but that other species such as *H. rufobrachium* play important roles in transmission when in areas co-occurring with *anerythrus*. Of great importance is the distinction between reservoir hosts that are able to sustain MPXV transmission and other host species that are infected but do not actually help maintain MXPV [40,41]. Unfortunately, this distinction is challenging to make due to difficulties in determining thresholds for persistence in host populations and estimating transmission rates between species [41]. Our study cannot specifically determine which species maintain the virus, however certain characteristics of *F. anerythrus* and some of our findings make it a strong candidate for taking part in maintaining MPXV transmission. *F. anerythrus* is typically found in higher abundances than other *Funisciurus* species and is thought to be better at colonizing areas due to its ability to swim and opportunistically forage in both arboreal and terrestrial habitats [39]. Additionally, species-specific social behaviors may affect MPXV transmission among host species. For example in Gabon, *F. anerythrus* are often seen in close pairs that travel together

and groom each other while sitting in contact. In contrast, *F. lemniscatus* are often in groups but usually maintain 5 to 20-m spacing while *F. pyrropus* are solitary [39]. Lower prevalence levels in both *lemniscatus* and *pyrropus* could be due to these behavioral differences that may limit intraspecific MPXV transmission. Additionally, co-occurrence of MPXV-positive samples for two species during the same year and in the same locality was seen on four occasions. All four involved *F. anerythrus*, supporting the possibility that multiple squirrel species may be infected with the virus within overlapping ranges but *anerythrus* acts as the main host species in maintaining MPXV. MPXV-positive *F. anerythrus* samples overlapped in locality with *F. congicus* positives in 1921and 1937 in Kunungu and in 1923 at the Mission of St Joseph Luluabourg. In 1960, the town of Lima had co-occurrences of *F. anerythrus* and *F. lemniscatus* positives. Furthermore, the opposite effect of *congicus* presence/absence on *anerythrus* MPXV-positive counts was not seen. This suggests that *anerythrus* is an important host that may play a main role in maintaining the virus, however it is hard to determine if this is in conjunction with other host species and if such a maintenance community varies across space.

Previous studies using ecological niche modeling methods predicted similar spatial distribution of MPXV as that seen in this study [42–46]. These models were trained on human case data and predicted suitable MPXV regions from environmental variables such as precipitation, temperature, tree cover, NDVI, soil pH/moisture—variables that coincide with humid lowland evergreen tropical forests that are good habitat for rope squirrels [42–46]. This suggests that environmental limitations in conjunction with host species distribution together shape the spatial distribution of MPXV infection. However, it is difficult to say if negatives of *congicus* from Angola are due to host community composition (no co-occurrence of *anerythrus*), environmental

limitations, or from museum differences alone. In general there is a transition from forest habitat in the DRC to woody savanna in Angola [47]; this transition could generally mean that squirrel densities in Angola are lower in addition to decreased richness in host community composition. Unfortunately, all the Angola specimens are from the AMNH and on average, the AMNH specimens were much less likely to be found positive for MPXV. Additional sampling of museum specimens for both the DRC and Angola is needed to determine if the *F. congicus* MPXV levels are truly this different across their range and to help guide theories on what species may be needed to maintain MPXV transmission.

The implications of this study for predicting risk for MPXV spillover into human populations are important, especially with increased pressure from deforestation and bushmeat hunting on the Congo Basin. Generally *Funisciurus* and *Heliosciurus* occupy all forest habitats, but oftentimes are seen as agricultural pests because they prefer degraded forest and agricultural habitats that border human habitations [13,14,39,48]. In a study in Equatorial Guinea, *Funisciurus* populations did not suffer any ill effect from hunting or agricultural disturbance, but actually increased in abundance in areas with more degraded habitat [49]. Furthermore, in much of the Congo Basin, bushmeat remains a primary source for protein, and Central African rodents are widely harvested in the region [48,50,51]. Past studies have shown that with continued pressure on mammal populations from bushmeat hunting, the decreased availability of larger mammals has resulted in hunters turning to rodent populations for sustenance [48,52]. As pressures from bushmeat hunting, deforestation, and agricultural activity increase, the risk of MPXV will likely also increase with elevated contact rates with infected host species through hunting and pest management of agricultural areas.

21

This study has shown the value of using museum collections to retrospectively examine pathogen distribution and temporal patterns. We have successfully amplified viral DNA, and the age of specimen was not correlated with lower MPXV prevalence. However, this study also suggests that the ability to amplify disease agents that occur at very low copy number may differ widely between museums due to different preservation and storage techniques. Ancient DNA may offer a valid method for understanding the historical presence of DNA viruses, however the use of museum specimens also comes with its own caveats. Overall, the MPXV prevalence levels estimated from museum specimens should be interpreted with an appropriate amount of caution due to the common issues inherent of studies that rely completely on museum samples. Successful DNA amplification is affected by various inconsistencies related to specimen preparation, preservation, and also sample collection [18,53,27,54]. Museum collectors and museums used various and sometimes unsystematic methods for specimen preparation, preservation, and storage over the years, including chemical treatments with arsenic that may inhibit both the enzymes used in DNA extraction and PCR [53]. Additionally, museum studies fall victim to sampling bias, especially in countries such as the DRC where samples are often limited to areas that are more accessible. Furthermore, our samples suffer from limited spatial resolution in terms of precise localities. In addition to uneven sampling spatially and limited spatial resolution, temporal shifts in sampling effort are evident and may change dramatically from year to year, especially in African countries where governmental status and stability can affect collecting surveys. For instance, 789 of 949 (83.1%) samples from the DRC were collected prior to the independence of the Belgian Congo in 1961. Unfortunately, sampling coverage in more recent years is poor, likely due to political unrest and dangerous field

conditions, making it difficult to examine temporal changes in MPXV infection in host species within the context of smallpox eradication and waning smallpox vaccination cross-immunity.

Other factors may have also influenced the MPXV screening in specimens and prevalence levels. Skin tissue has proved to be fairly useful for DNA amplification for various animals infected by MPXV [18], however viral DNA may be differentially sequestered in the host body depending on the transmission pathway, specific immunological host response, or viral targeting of host cells, especially in orthopoxviruses generally characterized by various forms of generalized vesiculopustular rashes and skin lesions [55]. Furthermore, little is understood about how long viral DNA persists in the skin after MPXV infection; the presence of viral DNA within the skin may be indicative of active infections or could also be a measure of past infection. In an acute infection such as MPXV, it is unlikely that our high prevalence levels are indicative of active infections. Additionally, our samples were not always taken from the same parts of the skin and this may affect ability to detect MPXV. Furthermore, the MPXV amplicons created by Li *et al.* [20] are based on all available human orthopoxvirus genomes; this presents the possibility that the amplified region may actually be more diverse within host species and our primers may not efficiently anneal to areas with disparities in sequence. If this is the case, we could be systematically missing strains that vary across space for which there are no human isolates. For example, our lack of positives in Angola may be attributed to the lack of human isolates for these areas and a systematic bias in our method of detection. Finally, our conservative measure of verifying positives through sequencing means that we could be missing many putative positives. In general, the low copy number of pathogen DNA in comparison to host DNA complicates the process of amplifying MPXV DNA [18].

23

We have taken multiple measures to avoid contamination; however there is the possibility that some positives in our study result from wild or PCR contamination. Following five of Cooper and Poinar's "nine criteria of authenticity" [25,26] does not exclude the possibility of cross-contamination from museum specimens while in storage prior to analyses [18]. Wild contamination after sample collection is unlikely as the copy number of viral DNA is already quite low; contamination would result in further dilution of these DNA particles, making it far more difficult to not only PCR amplify but also sequence these contaminants (Fig S1). As expected, the amplification of G2R_WA (101-bp) resulted in higher MPXV prevalence rates because its length was shorter than the other amplicon G2R_F (123-bp). While a 22-bp difference may not seem like a large length difference, the further complications of low copy number of viral DNA within the more highly concentrated DNA of the host species and stochastic amplification at low copy numbers could account for this difference during RT-PCR. Based on our Bioanalyzer results of the DNA extracts (both host and pathogen DNA), the difference in DNA concentration between a 100-bp and a 125-bp amplicon is about a 6.2% higher concentration for the shorter amplicon. In addition, stochastic effects in real-time PCR reactions are observed when less than 10 copies of the target DNA are present in the reaction [23]. Estimates of MPXV prevalence in skin specimens could be higher than actual cases if any contamination did occur. However, there is also the possibility that our estimates are lower than in reality if our methods for detection were not sensitive enough to pick up low copy DNA fragments. Viral DNA occurring at low copy numbers stochastically amplifies and could be dramatically different between specimens depending on the infection level or state of the animal at death.

**CONCLUSION**

This study has not only identified new potential hosts of MPXV, but it has also quantified relative differences between these hosts. These data allow us to build on the human risk predictions of previous modeling efforts [42–46] and create a more informed understanding of what areas are at higher risk of MPVX infection based on the actual ecology and infection rates of host species. This has implications for targeting MPXV surveillance and management. While this study focuses on the historical distribution and prevalence of MPXV infection, it helps inform how to target disease management efforts and resources to areas most affected by these specific host species.

Still unanswered is the question of MPXV prevalence throughout the range of *F. congicus* and if the lack of positives in Angola is a true signature of MPXV prevalence or is an artifact of museum DNA quality or primer design. Further testing of museum specimens from the DRC and Angola is needed to answer this question historically; but more importantly, large gaps in our knowledge remain with regards to the current distribution and prevalence levels of MPXV within infectious host populations and which species are essential for MPXV maintenance. A recent study uncovered four lineages of MPXV and patterns of gene loss within human MPXV genomic diversity [56]. Further work that examines the genetic diversity and population structure of MPXV within historical host populations could elucidate transmission links between host species, give insight into MPXV evolution and spillover into human populations, and allow for the distinction between reservoir hosts from other non-maintaining populations.

As the pressures of agriculture, deforestation, bushmeat hunting, and other anthropogenic activities increase, human contact rates with infected host species, such as the squirrels examined in this study, will likely increase. Our results suggest that future surveillance of MPXV within host populations should target *F. anerythrus* and *congicus* due to their broad spatial distribution throughout the DRC and the likelihood of their involvement in the maintenance of MPXV. Further information concerning general host ecology, such as abundance with respect to habitat type, and contact rates between human populations and squirrel populations would be valuable for estimating human risk and understanding the contribution of spillover from rope squirrels to the increase in MPXV human infections.
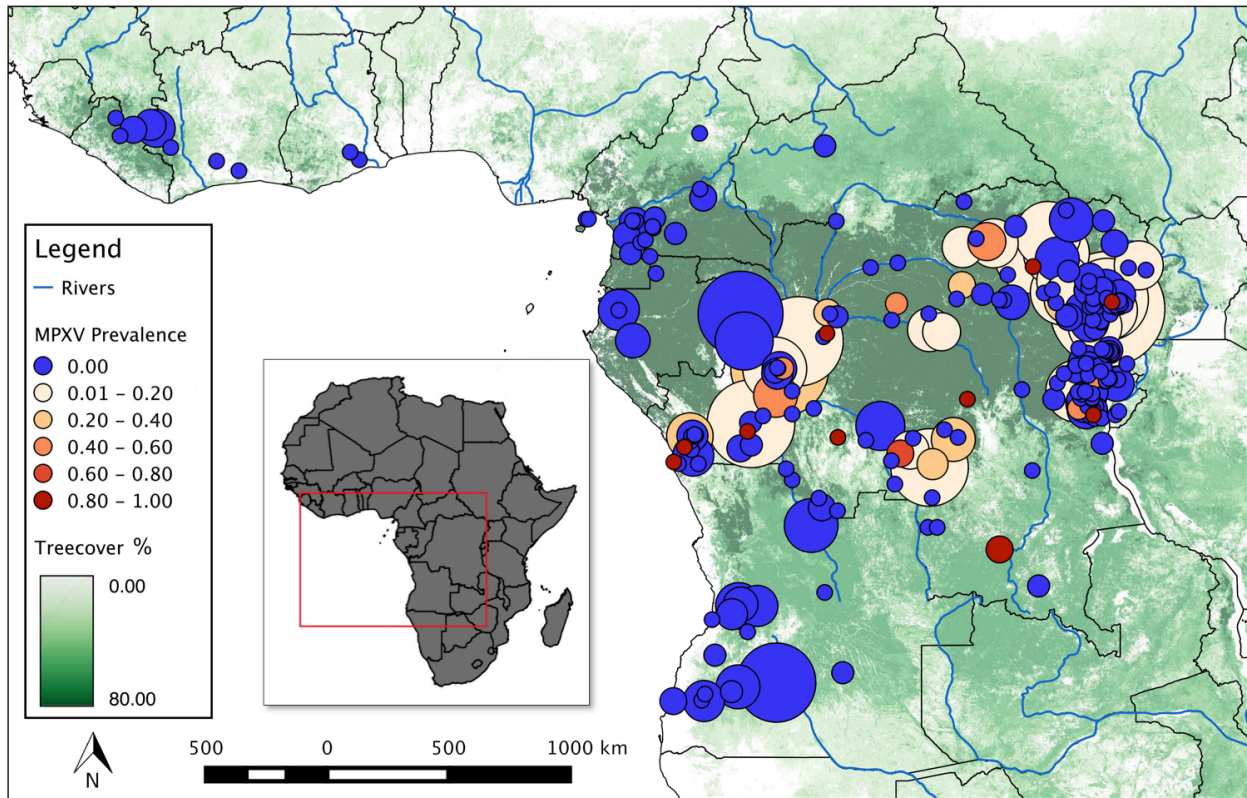
**Fig 1. Map of localities of *Funisciurus* samples and corresponding MPXV prevalence**
Localities and corresponding prevalence levels of museum skin samples positive for MPXV viral DNA for either the G2R_G or G2R_WA amplicon. The size of dots corresponds to the number of samples per locality, while color corresponds to a particular range of MPXV positive prevalence levels: blue for negatives, light reds for low MPXV prevalence, and darker reds for highly positive locations. These sampling locations are set on a tree cover layer with darker greens corresponding to high percentages of tree cover [57,58]. Localities are as exact as possible as given by museums; locality coordinates vary from town-level to province level. At larger spatial extents, the dot is geo-referenced to the centroid latitude and longitudes. Map created using QGIS Version 2.6.0-Brighton [34].
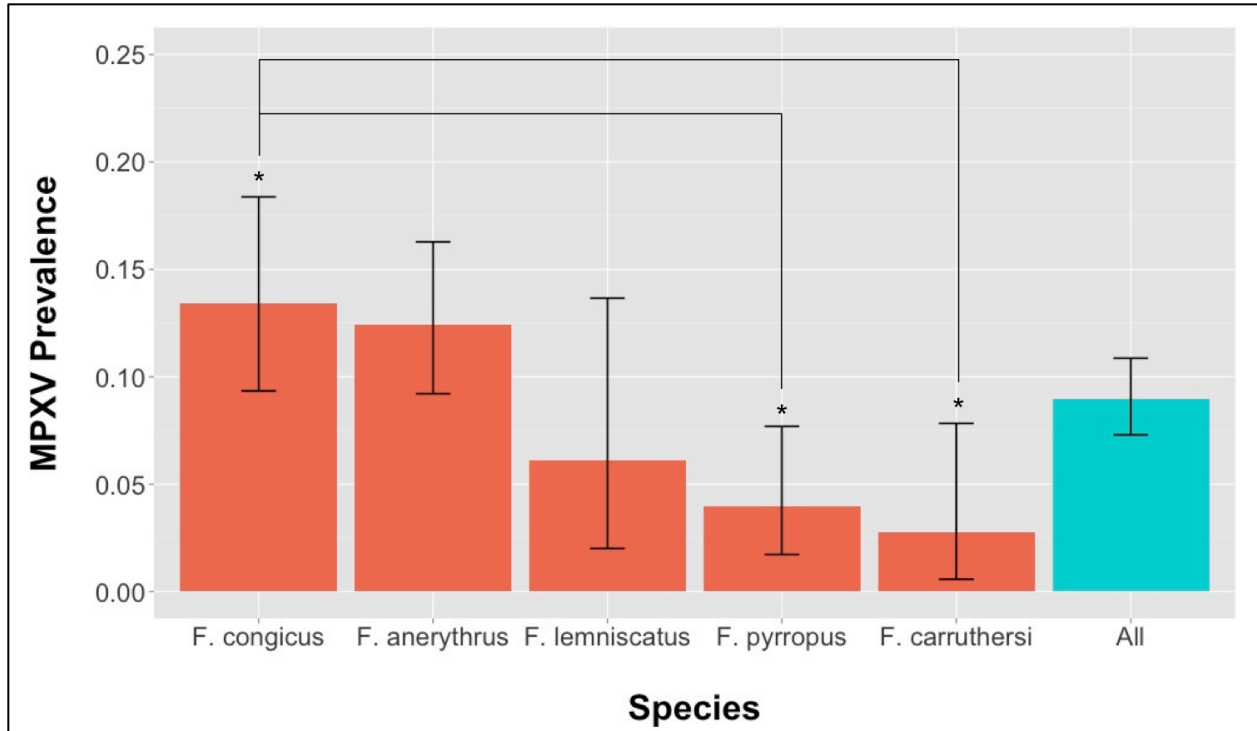
**Fig 2. Comparison of MPXV prevalence among skin specimens**
The total prevalence level for all sample specimens is shown along with prevalence levels for all
positive species: *Funisciurus congicus*, *Funisciurus anerythrus*, *Funisciurus lemniscatus*,
*Funisciurus pyrropus*, and *Funisciurus carruthersi*. Error bars are the Clopper-Pearson exact
95% confidence intervals. Tukey's pairwise differences in MPXV prevalence levels between
species were calculated, and asterisks denote the significant pairwise comparisons (* $p < 0.05$).
The results include positives for the two different amplicons (G2R_G and G2R_WA) tested; a
sample is considered positive for having successful amplification of either amplicon. For a more
specific breakdown by amplicon, see Fig S4 and Table 1. Figure created using R package
ggplot2 [29,59].
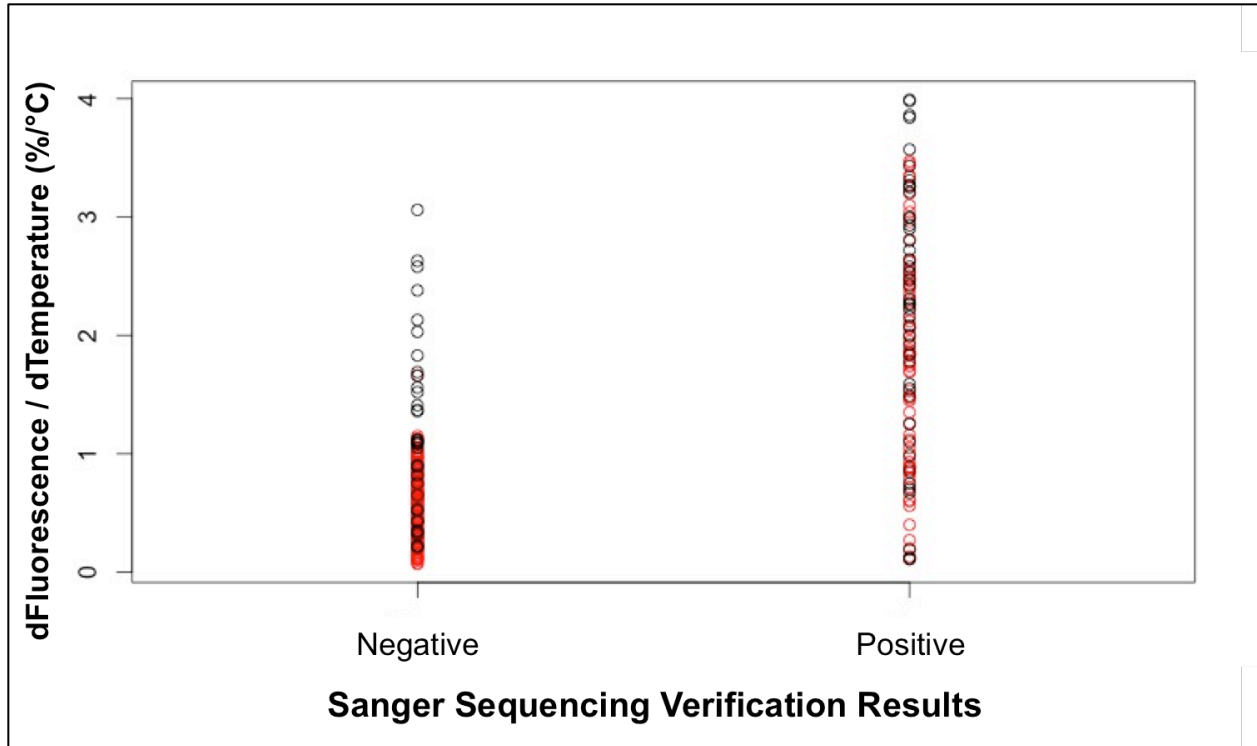
**Fig S1. Comparison of Sanger sequencing verification results to fluorescence levels of HRM putative positives**

Rate of fluorescence or the intensity of the $T_m$ peak as calculated through analysis of HRM melting curves acts as a proxy for copy number of PCR product [60]. Black circles correspond to the G2R_G amplicon and red points to the G2R_WA amplicon. This figure compares the sensitivity of HRM to that of Sanger sequencing. HRM putative positives melting in the correct melting range are more likely to be verified by Sanger-sequencing if the copy number is higher. This restricts the positives to those that occur in higher copy number, avoiding wild contamination. An ANOVA rejects the null hypothesis that the mean $T_m$ peak intensity of fluorescence for Sanger-sequenced positives is equal to the mean for Sanger-sequenced negatives ($p < 2.2E-16$, $F = 190.7$, $df = 1$).
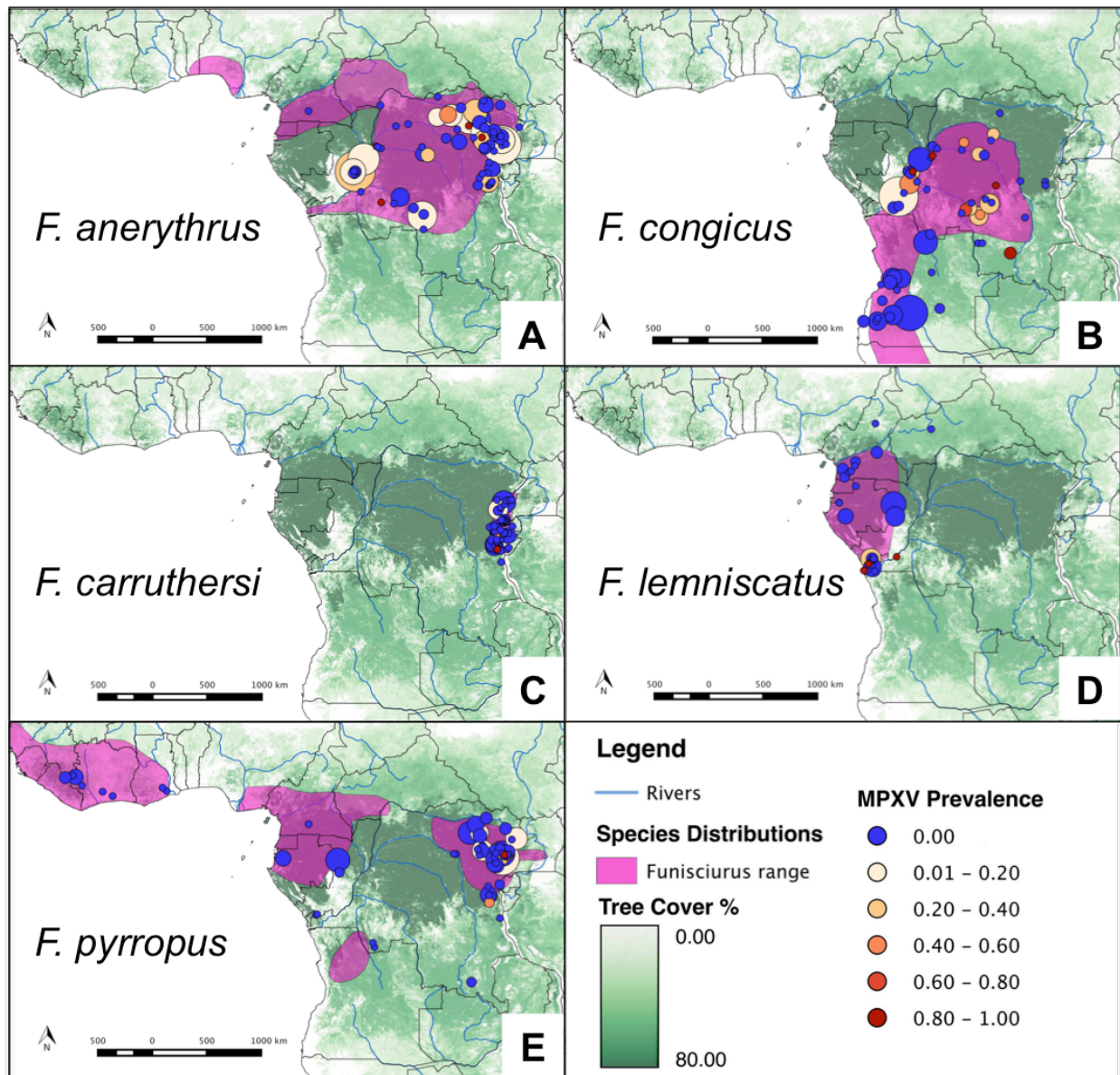
**Fig S2. MPXV Prevalence across localities for MPXV positive species**
MPXV prevalence across localities for MPXV positives species: (A) *F. anerythrus*, (B) *F. congicus*, (C) *F. carruthersi*, (D) *F. lemniscatus*, and (E) *F. pyrropus*. Prevalence levels are shown through coloration of dots with negatives as blue, slightly positive as light reds, and highly positive as dark red. Size of dote corresponds to the sampling number per locality. Estimated species ranges are shown in magenta [33]. These sampling locations are set on a tree cover layer with darker greens corresponding to high percentages of tree cover [57,58]. Localities are as exact as possible as given by museums; locality coordinates vary from town-level to province level. At larger spatial extents, the dot is geo-referenced to the centroid latitude and longitudes. Map created using QGIS Version 2.6.0-Brighton [34].
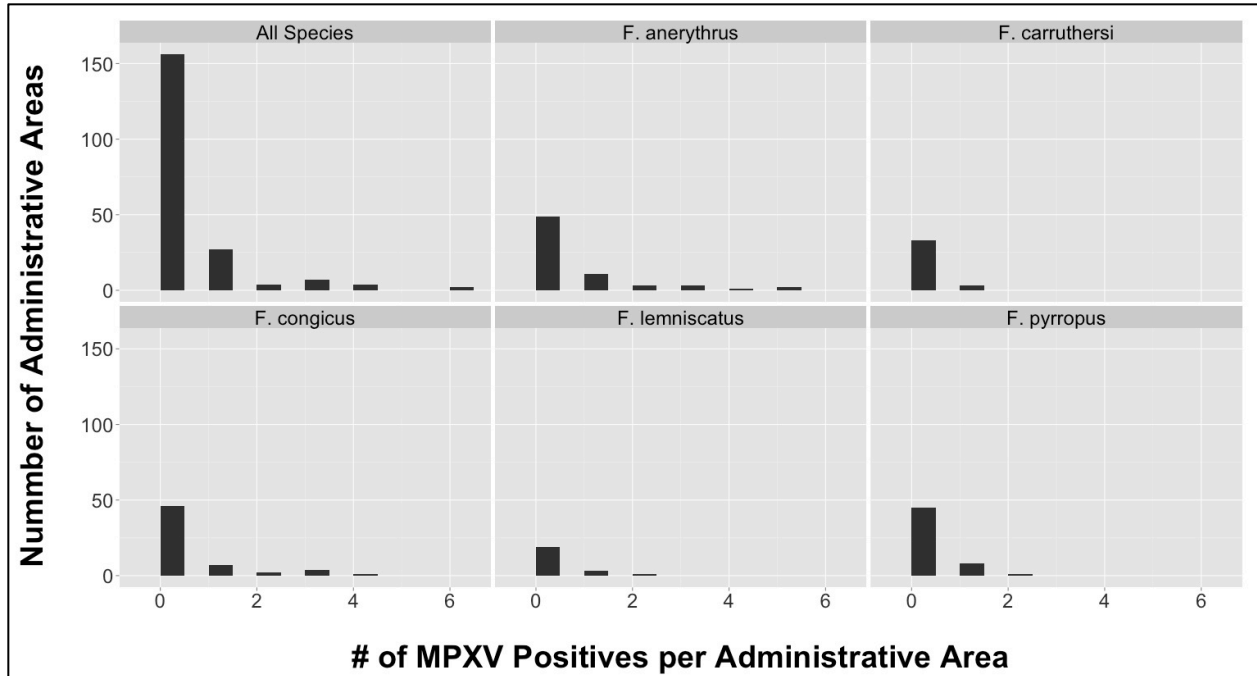
**Fig S3. Counts of MPXV Positives per Administrative Area for Positive Species**
Counts of MPXV positives per administrative area are compared to number of administrative area with that number of MPXV cases. These MPXV cases are shown for all species aggregated and the five MPXV positive species: *Funisciurus anerythrus*, *F. carruthersi*, *F. congicus*, *F. lemniscatus*, and *F. pyrropus*. Figure created using R package ggplot2 [29,59].
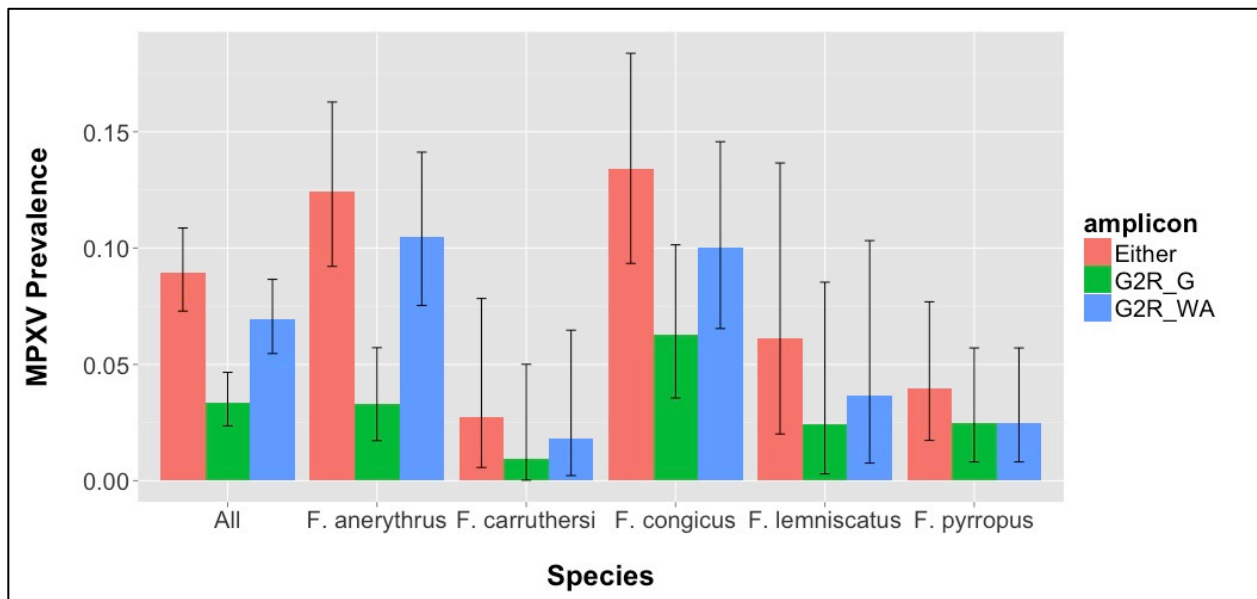


**Fig S4. Comparison of MPXV prevalence among skin specimens for both amplicons**
MPXV prevalence levels and their Clopper-Pearson exact 95% confidence intervals are shown for the (i) G2R_G amplicon (red), (ii) G2R_WA amplicon (green), and (iii) either amplicon (blue). MPXV prevalence levels are given overall and for the five MPXV positive species: *Funisciurus anerythrus*, *F. carruthersi*, *F. congicus*, *F. lemniscatus*, and *F. pyrropus*. Figure created using R package ggplot2 [29,59].
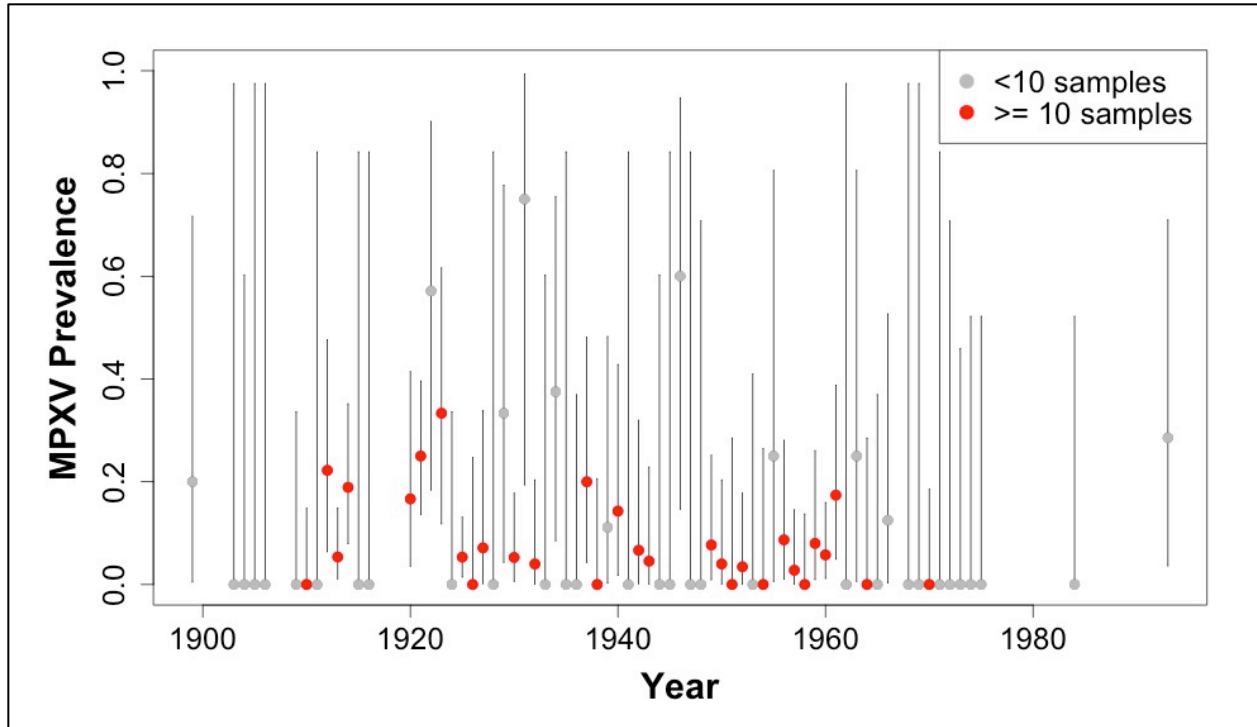
**Fig S5. MPXV Prevalence by year of collection for all species**
MPXV Prevalence by year of collection is shown for all species aggregated. All samples without a collection year were dropped from the dataset. Error bars are the Clopper-Pearson exact 95% confidence intervals. Sample sizes for the year below ten are represented in gray while years with ten or more samples are represented with red dots to highlight years with larger sample sizes.
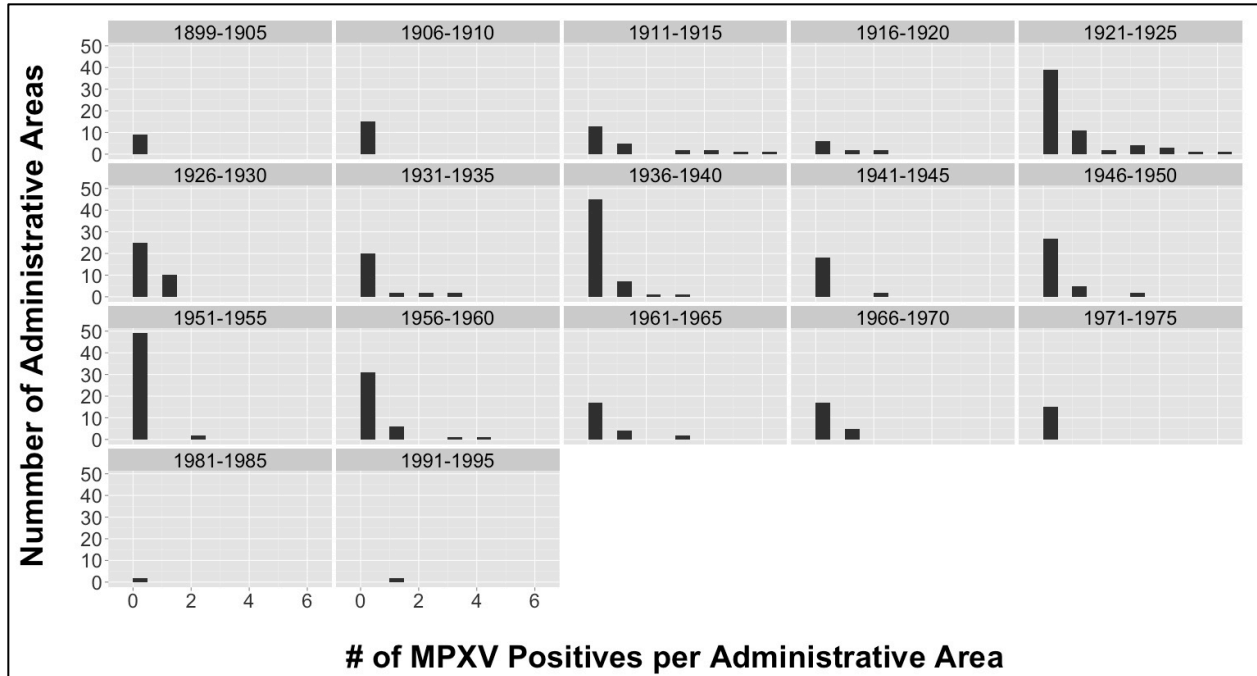
**Fig S6. Count of MPXV Positives by year of collection for all species**
Counts of MPXV positives per administrative area are compared to number of administrative area with that number of MPXV cases. These MPXV cases are shown across years binned by approximately 5 years. Figure created using R package ggplot2 [29,59].
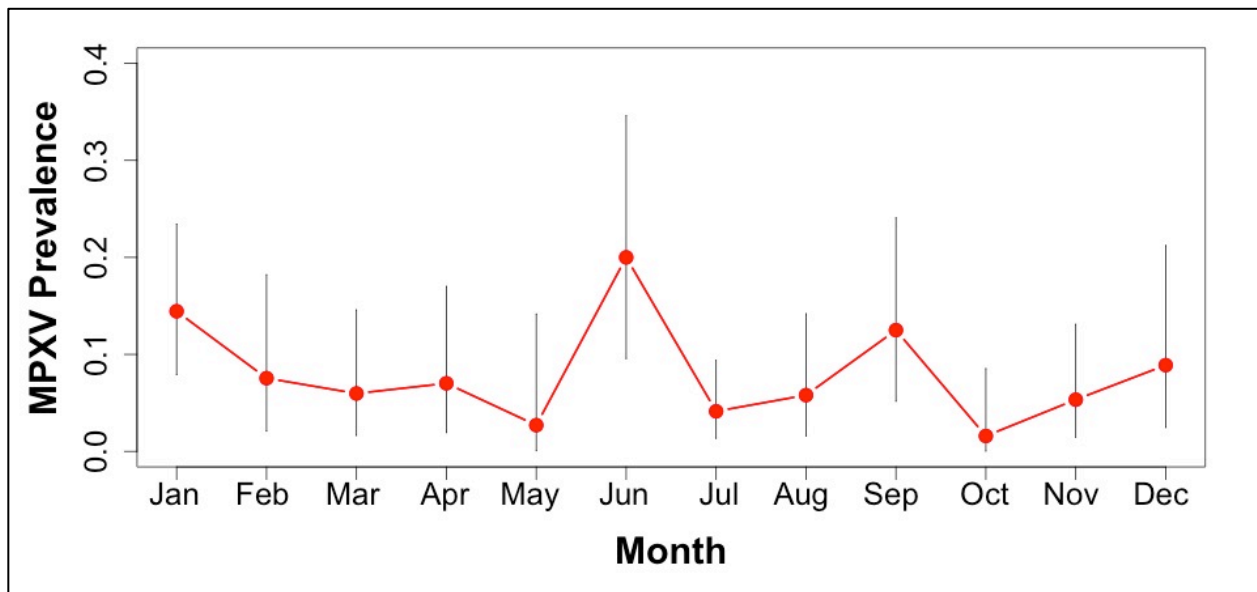


**Fig S7. MPXV Prevalence by month of collection for all years and species**
MPXV prevalence rates are given by month of collection for all years and species. All samples without a collection month were dropped from the dataset. Error bars are the Clopper-Pearson exact 95% confidence intervals.

# APPENDIX B: TABLES

**Table 1. MPXV-positive samples by species and comparison across amplicons**
Sample size for each species, number of positives, and MPXV prevalence of positive samples for (i) G2R_G amplicon, (ii) G2R_WA amplicon, or (iii) either amplicon with Clopper-Pearson exact 95% confidence intervals.

| Species Funisciurus | No. Tested | NO. POSITVE | | | MPXV PREVALENCE | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | G2R_G | G2R_WA | Either | G2R_G | 95% CIª | G2R_WA | 95% CIª | Either | 95% CIª |
| F. anerythrus | 362 | 12 | 38 | 45 | 0.033 | 0.017-0.057 | 0.1 | 0.075-0.14 | 0.12 | 0.092-0.16 |
| F. bayoni | 7 | 0 | 0 | 0 | 0 | 0-0.41 | 0 | 0-0.41 | 0 | 0-0.41 |
| F. carruthersi | 109 | 1 | 2 | 3 | 0.0092 | 0.00023-0.050 | 0.018 | 0.0022-0.065 | 0.028 | 0.0057-0.078 |
| F. congicus | 239 | 15 | 24 | 32 | 0.063 | 0.036-0.10 | 0.1 | 0.065-0.15 | 0.13 | 0.093-0.18 |
| F. isabella | 18 | 0 | 0 | 0 | 0 | 0-0.19 | 0 | 0-0.19 | 0 | 0-0.19 |
| F. lemniscatus | 82 | 2 | 3 | 5 | 0.024 | 0.0030-0.085 | 0.037 | 0.0076-0.10 | 0.061 | 0.020-0.14 |
| F. leonis | 2 | 0 | 0 | 0 | 0 | 0-0.84 | 0 | 0-0.84 | 0 | 0-0.84 |
| F. leucogenys | 6 | 0 | 0 | 0 | 0 | 0-0.46 | 0 | 0-0.46 | 0 | 0-0.46 |
| F. pyrropus | 201 | 5 | 5 | 8 | 0.025 | 0.0081-0.057 | 0.025 | 0.0081-0.057 | 0.04 | 0.017-0.077 |
| F. substriatus | 1 | 0 | 0 | 0 | 0 | 0-0.98 | 0 | 0-0.98 | 0 | 0-0.98 |
| F. spp. | 11 | 0 | 0 | 0 | 0 | 0-0.28 | 0 | 0-0.28 | 0 | 0-0.28 |
| TOTAL | 1038 | 35 | 72 | 93 | 0.034 | 0.024-0.047 | 0.069 | 0.055-0.087 | 0.09 | 0.073-0.11 |

ª Clopper-Pearson exact 95% confidence interval for binomial success probability, calculated using R package Hmisc version 3.14-6 [29,61].

REFERENCES:

1.  Arita I, Jezek Z, Khodakevich L, Ruti K. Human monkeypox: A newly emerged orthopoxvirus zoonosis in the tropical rain forests of Africa. Am J Trop Med Hyg. 1985;34: 781–789.

2.  Breman JG, Ruti K, Steniowski M V., Zanotto E, Gromyko AI, Arita I. Human monkeypox, 1970-79. Bull World Health Organ. 1980;58: 165–182.

3.  Jezek Z, Grab B, Szczeniowski M, Paluku KM, Mutombo M. Clinico-epidemiological features of monkeypox patients with an animal or human source of infection. Bull World Health Organ. 1988;66: 459–64. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2491168&tool=pmcentrez&rendertype=abstract

4.  Breman JG. Monkeypox: an Emerging Infection for Humans? In: Scheld WM, Craig WA, Hughes JM, editors. Emerging Infections 4. Washington, D.C.: ASM Press; 2000. pp. 45–67.

5.  Jezek Z, Fenner F. Monographs in Virology: Human Monkeypox. Melnick JL, editor. Basel, Switzerland: S. Karger AG; 1988.

6.  Arita I, Henderson D a. Smallpox and monkeypox in non-human primates. Bull World Health Organ. 1968;39: 277–283.

7.  Rimoin AW, Mulembakani PM, Johnston SC, Lloyd JO, Kisalu NK, Shongo RL, et al. Major increase in human monkeypox incidence 30 years after smallpox vaccination campaigns cease in the Democratic Republic of Congo. Proc Natl Acad Sci U S A. 2010;107: 16262–16267. doi:10.1073/pnas.1005769107/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1005769107

8.  Jezek Z, Arita I, Mutombo M, Dunn C, Nakano JH, Szczeniowski M. Four generations of probable person-to-person transmission of human monkeypox. Am J Epidemiol. 1986;123: 1004–1012.

9.  Lloyd-Smith JO. Vacated niches, competitive release and the community ecology of pathogen eradication. Philos Trans R Soc Lond B Biol Sci. 2013;368: 20120150. doi:10.1098/rstb.2012.0150

10. Khodakevich L, Jezek Z, Messinger D. Monkeypox virus: ecology and public health significance. Bull World Health Organ. 1988;66: 747–752.

11. Hutin YJ, Williams RJ, Malfait P, Pebody R, Loparev VN, Ropp SL, et al. Outbreak of human monkeypox, Democratic Republic of Congo, 1996 to 1997. Emerg Infect Dis. 2001;7: 434–8. doi:10.3201/eid0703.010311

12. Khodakevich L, Jezek Z, Kinzanaka K. Isolation of monkeypox virus from wild squirrel infected in nature. Lancet. 1986;9? 98–99.

13. Khodakevich L, Szczeniowski M, Manbu-ma-Disu, Jezek Z, Marennikova S, Nakano J, et al. The role of squirrels in sustaining monkeypox virus transmission. Trop Geogr Med. 1987;39: 115–122.

14. Khodakevich L, Szczeniowski M V, Nambu-ma-Disu, Jezek Z, Marennikova S, Nakano J, et al. Monkeypox virus in relation to the ecological features surrounding human settlements in Bumba zone, Zaire. Trop Geogr Med. 1987;39: 56–63.

15. Reynolds MG, Carroll DS, Olson V a., Hughes C, Galley J, Likos A, et al. A silent enzootic of an orthopoxvirus in Ghana, West Africa: Evidence for multi-species involvement in the absence of widespread human disease. Am J Trop Med Hyg. 2010;82: 746–754. doi:10.4269/ajtmh.2010.09-0716

16. Hutson CL, Lee KN, Abel J, Carroll DS, Montgomery JM, Olson V a., et al. Monkeypox zoonotic associations: Insights from laboratory evaluation of animals associated with the multi-state US outbreak. Am J Trop Med Hyg. 2007;76: 757–767.

17. Suarez A V., Tsutsui ND. The Value of Museum Collections for Research and Society. Bioscience. 2004;54: 66. doi:10.1641/0006-3568(2004)054[0066:TVOMCF]2.0.CO;2

18. Tsangaras K, Greenwood AD. Museums and disease: using tissue archive and museum samples to study pathogens. Ann Anat. Elsevier GmbH.; 2012;194: 58–73. doi:10.1016/j.aanat.2011.04.003

19. McCollum AM, Li Y, Wilkins K, Karem KL, Davidson WB, Paddock CD, et al. Poxvirus viability and signatures in historical relics. Emerg Infect Dis. 2014;20: 177–84. doi:10.3201/eid2002/131098

20. Li Y, Zhao H, Wilkins K, Hughes C, Damon IK. Real-time PCR assays for the specific detection of monkeypox virus West African and Congo Basin strain DNA. J Virol Methods. 2010;169: 223–227. doi:10.1016/j.jviromet.2010.07.012

21. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3-new capabilities and interfaces. Nucleic Acids Res. 2012;40: 1–12. doi:10.1093/nar/gks596

22. Koressaar T, Remm M. Enhancements and modifications of primer design program Primer3. Bioinformatics. 2007;23: 1289–1291. doi:10.1093/bioinformatics/btm091

23. Morrison TB, Weis JJ, Wittwer CT. Quantification of lowcopy transcripts by continuous SYBR® green I monitoring during amplification. BioTechniques. 1998. pp. 954–962.

24. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012;28: 1647–1649. doi:10.1093/bioinformatics/bts199

25. Cooper A, Poinar HN. Ancient DNA: Do It Right or Not at All. Sci Lett. 2000;289: 1139b.

26. Gilbert MTP, Bandelt H-J, Hofreiter M, Barnes I. Assessing ancient DNA studies. Trends Ecol Evol. 2005;20: 541–4. doi:10.1016/j.tree.2005.07.005

27. Paabo S, Higuchi RG, Wilson AC. Ancient DNA and the Polymerase Chain Reaction: The Emerging Field of Molecular Archaeology. J Biol Chem. 1989;264: 9709–9712.

28. RStudio. RStudio: Integrated development environment for R [Internet]. Boston, MA; 2012. Available: http://www.rstudio.org

29. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2013.

30. Fox J, Weisberg S. An {R} Companion to Applied Regression [Internet]. Second. Thousand Oaks {CA}: Sage; 2011. Available: http://socserv.socsci.mcmaster.ca/jfox/Books/Companion

31. Hothorn T, Bretz F, Westfall P. Simultaneous inference in general parametric models. Biometrical J. 2008;50: 346–363. doi:10.1002/bimj.200810425

32. National Geospatial-Intelligence Agency. GEOnet Names Server database [Internet]. 2015. Available: http://geonames.nga.mil/namesgaz/

33. IUCN. IUCN Red List of Threatened Species [Internet]. 2012. Report No.: 2012.2. Available: http://www.iucnredlist.org

34. QGIS Development Team. QGIS Geographic Information System [Internet]. 2014. Available: http://qgis.osgeo.org

35. Hijmans RJ. raster: Geographic data analysis and modeling [Internet]. 2014. Available: http://cran.r-project.org/package=raster

36. Bivand R, Keitt T, Rowlingson B. rgdal: Bindings for the Geospatial Data Abstraction Library [Internet]. 2014. Available: http://cran.r-project.org/package=rgdal

37. Bates D, Maechler M, Bolker B, Walker S. lme4: Linear mixed-effects models using Eigen and S4 [Internet]. 2014. Available: http://cran.r-project.org/package=lme4/

38.    Bartoń K. MuMIn: Multi-Model Inference [Internet]. 2015. Available: http://cran.r-project.org/package=MuMIn

39.    Emmons LH. Ecology and resource partitioning among nine species of African rain forest squirrels. Ecol Monogr. 1980;50: 31–54. Available: <Go to ISI>://ZOOREC:ZOOR11700058652

40.    Haydon DT, Cleaveland S, Taylor LH, Laurenson MK. Identifying reservoirs of infection: A conceptual and practical challenge. Emerg Infect Dis. 2002;8: 1468–1473. doi:10.3201/eid0812.010317

41.    Viana M, Mancy R, Biek R, Cleaveland S, Cross PC, Lloyd-Smith JO, et al. Assembling evidence for identifying reservoirs of infection. Trends Ecol Evol. Elsevier Ltd; 2014;29: 270–279. doi:10.1016/j.tree.2014.03.002

42.    Thomassen H a, Fuller T, Asefi-Najafabady S, Shiplacoff J a G, Mulembakani PM, Blumberg S, et al. Pathogen-host associations and predicted range shifts of human monkeypox in response to climate change in central Africa. PLoS One. 2013;8: e66071. doi:10.1371/journal.pone.0066071

43.    Fuller T, Thomassen H a, Mulembakani PM, Johnston SC, Lloyd-Smith JO, Kisalu NK, et al. Using remote sensing to map the risk of human monkeypox virus in the Congo Basin. Ecohealth. 2011;8: 14–25. doi:10.1007/s10393-010-0355-5

44.    Levine RS, Peterson a T, Yorita KL, Carroll D, Damon IK, Reynolds MG. Ecological niche and geographic distribution of human monkeypox in Africa. PLoS One. 2007;2: e176. doi:10.1371/journal.pone.0000176

45.    Nakazawa Y, Lash RR, Carroll DS, Damon IK, Karem KL, Reynolds MG, et al. Mapping monkeypox transmission risk through time and space in the Congo Basin. PLoS One. 2013;8: e74816. doi:10.1371/journal.pone.0074816

46.    Ellis CK, Carroll DS, Lash RR, Peterson AT, Damon IK, Malekani J, et al. Ecology and geography of human monkeypox. J Wildl Dis. 2012;48: 335–347.

47.    Broxton PD, Zeng X, Sulla-Menashe D, Troch P a. A global land cover climatology using MODIS data. J Appl Meteorol Climatol. 2014;53: 1593–1605. doi:10.1175/JAMC-D-13-0270.1

48.    Carpaneto GM, Germi FP. Diversity of mammals and traditional hunting in central African rain forests. Agric Ecosyst Environ. 1992;40: 335–354. doi:10.1016/0167-8809(92)90101-G

49.    Rist J, Milner-gulland EJ, Cowlishaw G, Rowcliffe JM. The Importance of Hunting and Habitat in Determining the Abundance of Tropical Forest Species in Equatorial Guinea. Biotropica. 2009;41: 700–710.

50.    Wilkie DS, Carpenter JF. Bushmeat hunting in the Congo Basin: An assessment of impacts and options for mitigation. Biodivers Conserv. 1999;8: 927–955. doi:10.1023/A:1008877309871

51.    Amori G, Gippolit S. Rodents and the bushmeat harvest in Central Africa. In: Mainka S, Trivedi M, editors. Links between Biodiversity Conservation, Livelihoods and Food Security: The sustainable use of wild species for meat. Gland, Switzerland: IUCN; 2002. pp. 95–100.

52.    Fa JE, Ryan SF, Bell DJ. Hunting vulnerability, ecological characteristics and harvest rates of bushmeat species in afrotropical forests. Biol Conserv. 2005;121: 167–176. doi:10.1016/j.biocon.2004.04.016

53.    Hall LM, Willcox MS, Jones DS. Association of enzyme inhibition with methods of museum skin preparation. Biotechniques. 1997;22: 928–934.

54.    Hofreiter M, Serre D, Poinar HN, Kuch M, Pääbo S. Ancient DNA. Nat Rev Genet. 2001;2: 353–359. doi:10.1038/35072071

55.    Chapman JL, Nichols DK, Martinez MJ, Raymond JW. Animal models of orthopoxvirus infection. Vet Pathol. 2010;47: 852–870. doi:10.1177/0300985810378649

56.    Kugelman JR, Johnston SC, Mulembakani PM, Lee MS, Koroleva G, Mccarthy SE, et al. Genomic Variability of Monkeypox Virus among Humans , Democratic Republic of the Congo. Emerg Infect Dis. 2014;20: 1–17.

57.    DeFries R, Hansen M, Townshend JRG, Janetos AC, Loveland TR. 1 Kilometer Tree Cover Continuous Fields 1.0. College Park, Maryland: Department of Geography, University of Maryland; 2000.

58.    DeFries R, Hansen M, Townshend JRG, Janetos AC, Loveland TR. A new global 1km data set of percent tree cover derived from remote sensing. Glob Chang Biol. 2000;6: 247–254.

59.    Wickham H. ggplot2: elegant graphics for data analysis [Internet]. Springer New York; 2009. Available: http://had.co.nz/ggplot2/book

60.    Zhou L, Palais R a., Paxton CN, Geiersbach KB, Wittwer CT. Copy Number Assessment by Competitive PCR with Limiting Deoxynucleotide Triphosphates and High-Resolution Melting. Clin Chem. 2015;61: 724–733. doi:10.1373/clinchem.2014.236208

61.    Harrell FEJ, Dupont with contributions from C, Others. M. Hmisc: Harrell Miscellaneous [Internet]. 2015. Available: http://cran.r-project.org/package=Hmisc