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Squirrel reservoirs of monkeypox virus are sister species separated by the Sanaga River (Cameroon), as are the two main viral clades

Manon Curaudeau [a,b], Julian Kerbis Peterhans [c,d], Thomas Le Flanchec [a,b], Emmanuel Gilissen [e,f], Laurent Granjon [g], Lison Barale [a], Antoine Gessain [b], Alexandre Hassanin [a,*]

[a] Institut de Systématique, Évolution, Biodiversité (ISYEB), MNHN, CNRS, EPHE, UA, Sorbonne Université, Paris, France
[b] Unité Épidémiologie et Physiopathologie des Virus Oncogènes, Institut Pasteur, CNRS UMR 3569, Université Paris Cité, Paris, France
[c] College of Arts & Sciences, Roosevelt University; Chicago, IL, USA
[d] National Museums of Kenya, Nairobi, Kenya
[e] Department of African Zoology, Royal Museum for Central Africa, Tervuren, Belgium
[f] Alzheimer and Other Tauopathies Research Group, ULB Center for Diabetes Research (UCDR), Université Libre de Bruxelles, Brussels, Belgium
[g] Centre de Biologie pour la Gestion des Populations (CBGP), IRD, INRAE, CIRAD, Institut Agro, Univ Montpellier, Montpellier, France

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ABSTRACT

Monkeypox is an emerging infectious disease of unclear zoonotic origin in Africa although increasing evidence suggests that rope squirrels are reservoirs of the monkeypox virus (MPXV).

Here, we describe new data on the systematics of rope squirrels (Sciuridae, *Funisciurus*). Our molecular phylogeny strongly supports the polyphyly of *Funisciurus pyrropus*, with a Central African clade sister to *Funisciurus leucogenys* and a West African clade sister to *Funisciurus anerythrus*. Museum specimens show that the two clades have distinct pelage patterns. We propose therefore to resurrect the name *Funisciurus leucostigma* for the West African clade. Using reliable taxonomic identifications of georeferenced squirrels, we inferred the ecological niches of the three species of interest. The Sanaga River in Cameroon appears to be the barrier separating both the sister species *F. anerythrus* and *F. leucostigma* as well as the sister MPXV clades I and II. This suggests that these two MPXV clades were isolated in Central and West Africa, respectively, due to allopatric squirrel speciation.

1. Introduction

Monkeypox, also known as mpox, is an emerging infectious disease caused by the Monkeypox virus (MPXV). Taxonomically, MPXV belongs to the family *Poxviridae*, which contains large double-stranded DNA viruses (130–375 kbp), and to the genus *Orthopoxvirus*, which only includes poxviruses related to mammalian hosts, such as smallpox, vaccinia and cowpox. Typically, mpox affects both children and adults following three phases: incubation, prodrome, and the eruptive stage. The latter stage lasts from 2 to 4 weeks and produces skin lesions evolving from macules to papules, vesicles and then to pustules. The lesions develop crusts that desquamate. Symptom severity is proportional to the density of skin lesions. The disease affects children, pregnant women and patients with immunodeficiency more severely. Monkeypox usually has a self-limiting course, although scarring is common [1].

The first human mpox case was detected in 1970 in the northwest of the Democratic Republic of Congo (DRC) [2]. Since then, several sporadic cases and epidemics have been documented in human populations from Central and West Africa, with case fatality rates varying from 1 % to 15 % [1]. The number of cases per year has significantly increased since the 2000s, probably due to the end of smallpox vaccination which provided cross-protection against MPXV [3]. Two small outbreaks outside the African endemic regions have also been reported: one in the United States in 2003, linked to the importation of infected rodents from Ghana [4], and another in Sudan in 2005 [5]. Two major outbreaks occurred recently. The first, spanning 2022–2023, resulted in over 100,000 reported cases worldwide. It traces back to the 2017 outbreak in Nigeria and had caused limited international spread between 2018 and 2021, before escalating into a global outbreak in May 2022 involving mostly homosexual men [6,7]. The second outbreak has been ongoing since 2023: the virus was initially detected in South Kivu

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* Corresponding author.
E-mail address: alexandre.hassanin@mnhn.fr (A. Hassanin).

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(eastern DRC); in 2024, it spread, mainly by heterosexual transmission, to several other African countries, causing hundreds of cases per week [8,9].

The ecological niche of the MPXV shows a fragmented distribution that fits within the three main blocks of Afrotropical rainforests [10,11], including (i) the Upper Guinean forests (UGF) and (ii) Lower Guinean forests (LGF) in West Africa, and (iii) the Congo Basin in Central Africa. Such fragmentation of the MPXV distribution is concordant with the phylogenetic analyses of MPXV genomes, which supports a deep divergence between the Central African (clade I) and West African clades (clade II) and a more recent divergence in West Africa between subclades IIa (viruses from UGF countries, including Côte d'Ivoire, Ghana, Liberia, and Sierra Leone) and IIb (viruses from LGF countries, including Nigeria and Cameroon) [10,12,13].

Previous human cases or epidemics have suggested that MPXV can be transmitted through contact with animals in African rainforests [1]. Although MPXV has been detected in many mammal species, most are likely secondary hosts and the reservoir host has not yet been fully identified. Based on ecological niche comparisons between the MPXV and 99 mammal species, Thomas's rope squirrel, *Funisciurus anerythrus* (Thomas, 1890), was found to be the best candidate as reservoir host of the MPXV [11]. This finding was consistent with a series of previous studies linking MPXV to *F. anerythrus* in DRC (Central Africa): (i) the virus was isolated from a *F. anerythrus* specimen collected in Yambuku [14]; (ii) several independent teams have generated MPXV sequences from *F. anerythrus* collected in different localities [10,15,16]; and (iii) anti-*Orthopoxvirus* antibodies have been detected with seroprevalence up to 50 % in *F. anerythrus* [17,18]. A recent study in the Taï National Park in Côte d'Ivoire (West Africa) provided strong evidence of an epizootic disease in a group of wild sooty mangabeys (*Cercocebus atys*) due to cross-species transmission from a rope squirrel [19]. The authors identified this squirrel as *Funisciurus pyrropus* (F. Cuvier, 1833), as this is the only species of *Funisciurus* currently recognized in the forests of Côte d'Ivoire and neighbouring countries [20,21].

In this study, we investigated the systematics of rope squirrels (Sciuridae, Protoxerini, *Funisciurus*) based on mitochondrial sequences and morphological characters. Our analyses revealed that the squirrel recently identified as a MPXV reservoir in Côte d'Ivoire [19] does not belong to *F. pyrropus*, but to another species closely related to *F. anerythrus*. We found that this taxonomic result has important implications for our understanding of the co-evolution between MPXV and rope squirrels.

2. Material and methods

2.1. DNA extraction, amplification, and sequencing

DNA was extracted using the Qiagen QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) from tissues of squirrel specimens conserved in the following institutions (Appendix A): RMCA (Royal Museum for Central Africa, Tervuren, Belgium), FMNH (Field Museum of Natural History, Chicago, USA), MNHN (*Muséum national d'Histoire naturelle*, Paris, France), CBGP (*Centre de Biologie pour la Gestion des Populations* - Small mammal Collection, France, Montferrier-sur-Lez), MVZ (Museum of Vertebrate Zoology, Berkeley, USA), and ZFMK (Zoological Research Museum A. Koenig, Bonn, Germany). No live animals were handled for the purpose of this study and thus no ethical approval was necessary.

DNA samples obtained from the 13 tissues fixed in ethanol (12 *Funisciurus* and 1 *Heliosciurus*) were quantified with the Quibit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) on a Qubit 3.0 Fluorometer. The libraries and DNA sequencing were done at the *Institut du Cerveau et de la Moelle épinière* on a Novaseq6000 Illumina system using the NovaSeq 6000 SP Reagent Kit (500 cycles) (Illumina, San Diego, CA, USA). Complete mitochondrial genomes (about 16.5 kb) were reconstructed *de novo* with GetOrganelle v1.7.7.0 [22]. Trimmed reads were mapped using NextGenMap v0.5.5 [23] against the consensus with a percentage of identity of 99 %, to obtain the final sequence of the mitochondrial genomes.

DNA samples obtained from dried skin of 50 museum specimens identified as *F. pyrropus*, *F. anerythrus* and *F. leucogenys* (Appendix A) were amplified by PCR in a volume of 20 μl including 3 μl of Buffer 10× with MgCl2, 2 μl of dNTP (6.6 mM), 0.25 μl of Taq DNA polymerase (2.5 U, Qiagen, Hilden, Germany) and 0.75 μl of the two following *Cyb* primers at 10 μM: U219 (5'-GTMACMCACATCTGYCGAGAYGT-3') and L403 (5'-CCTCAGAANGATATTTGTCCTCA −3'). The standard PCR conditions were as follows: 4 min at 94 °C; 5 cycles of denaturation/annealing/extension with 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C, followed by 5 cycles of 30 s at 94 °C, 1 min at 55 °C and 1 min at 72 °C, and 30 cycles of 30 s at 94 °C, 1 min at 50 °C, and 1 min at 72 °C, followed by 7 min at 72 °C. The amplicons were sequenced with the Sanger method in both directions by Eurofins MWG Operon (Ebersberg, Germany). Haplotypes were assembled with forward and reverse electropherograms using Sequencher 5.1 (Gene Codes Corporation, Ann Arbor, MI, USA).

2.2. Phylogenetic analyses

The 13 mitochondrial genomes sequenced for this study (12 *Funisciurus* squirrels and *Heliosciurus gambianus*) were aligned to those of six outgroup species reconstructed from available SRA data (Appendix A). Mitogenomes were aligned using MAFFT 7.450 [24] using default parameters and refined by eyes on AliView 1.26 [25]. The final alignment of mitogenomes contains 16,539 bp. We added to this alignment all *Funisciurus* sequences available in GenBank for four mitochondrial genes, including 23 cytochrome *b* (*Cyb*), two cytochrome *c* oxidase subunit I (*CO1*), two 12S ribosomal RNA (*12S*) and two 16S ribosomal RNA (*16S*). We also included 50 *Cyb* fragments sequenced in this study from museum specimens of the collections of the RMCA (Royal Museum for Central Africa) and MNHN (*Muséum national d'Histoire naturelle*). Therefore, our mitochondrial dataset includes 95 taxa. Two datasets were used for the phylogenetic analyses: (i) the alignment of the 19 complete mitogenomes; (ii) and the mitochondrial dataset of 95 taxa. The maximum-likelihood (ML) analyses were performed in IQ-TREE2 [26] using 1000 bootstrap replicates and the best-fit model (TIM2 + F + I + G4).

The phylogeography of MPXV was inferred using an alignment of complete genomes. All the 564 MPXV genomes available in GenBank were downloaded and aligned using MAFFT 7.450 [24]. Then, we removed MPXV genomes showing more than 1 % of missing data in order to limit the impact of sequencing errors in our analyses. We also trimmed the 5′ and 3′ ends of the MPXV alignment as many tandem duplications posed serious problems in assessing primary homology. Three other species of *Orthopoxvirus* were used as outgroup to root the MPXV tree: *O. cowpox*, *O. ectromelia*, and *O. vaccinia*. Our final dataset includes 167 genomes and 201,242 bp. The ML tree was reconstructed under IQ-TREE2 [26] using 1000 bootstrap replicates and the best-fit model (K3Pu + F + I).

2.3. Ecological niche analyses

Three categories of occurrence records were used for inferring the ecological niches of each *Funisciurus* species: (i) specimens for which mitochondrial sequences were available in GenBank or were specially produced for this study; (ii) specimens examined in the MNHN collection for their coat coloration pattern and biometric measurements; and (iii) georeferenced squirrels photographed in the iNaturalist database [27]. Occurrence records of MPXV included animal cases from which the virus was isolated and/or sequenced, as well as human index cases presumed to be infected from an animal source and confirmed through PCR, DNA sequencing, or viral isolation. To reduce sampling bias and improve the performance of ecological niche models, occurrence records located less than 10 km apart were filtered out using spThin [28].

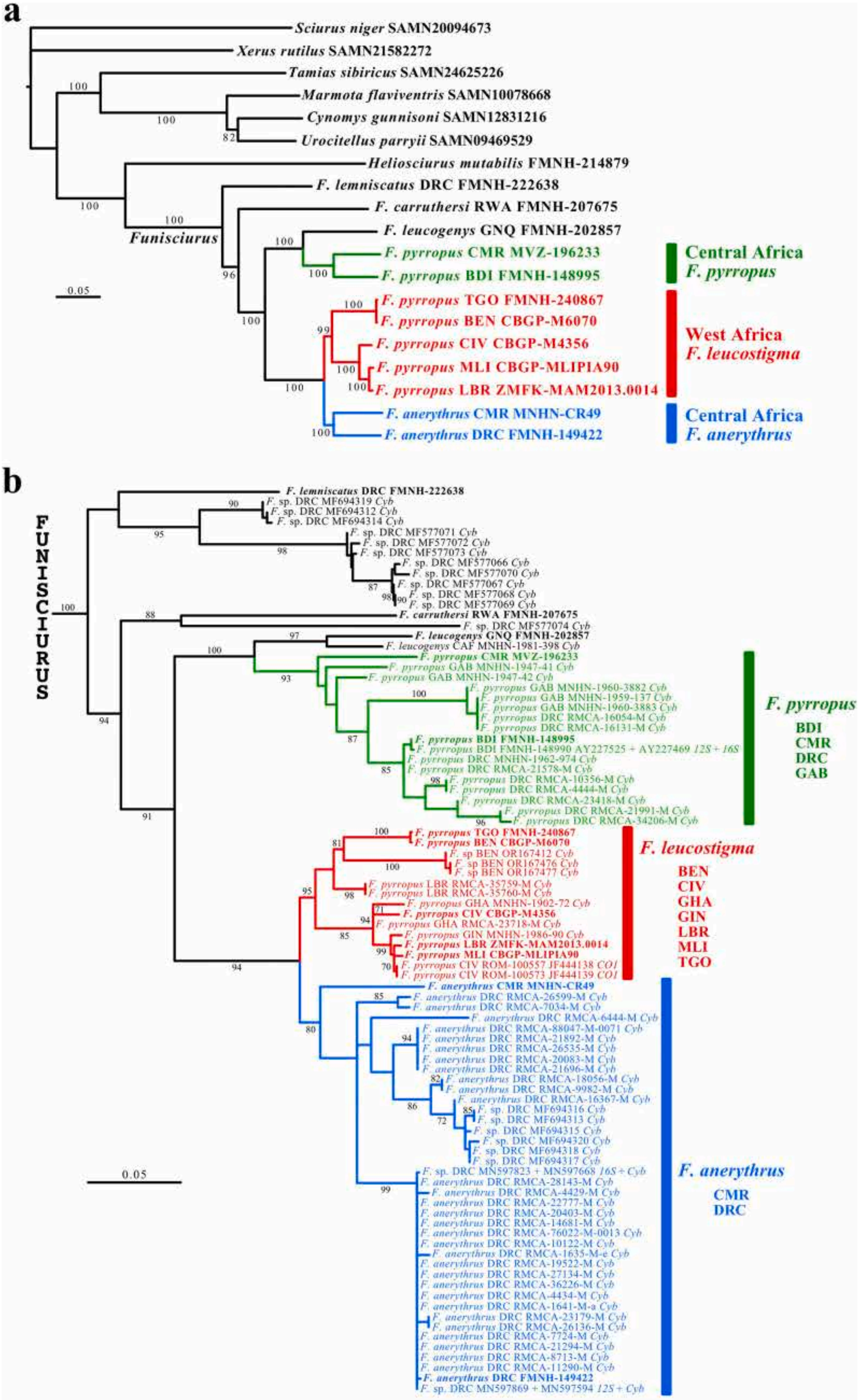

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Fig. 1. Molecular phylogeny of rope squirrels (genus *Funisciurus*) based on complete mitogenomes (a), and including additional specimens only sequenced for one or two mitochondrial genes, *i.e.*, cytochrome *b* (*Cyb*), cytochrome *c* oxidase subunit I (*CO1*), and 12S and 16S ribosomal RNA (*12S* and *16S*) (b).
The two datasets of 16,539 bp were analysed using the maximum-likelihood method. The values at the nodes are bootstrap percentages ≥70 %.
For each sequence in (b), we indicated the original species name, country code (see map of Fig. 3a), collection number and/or GenBank accession number (only for sequences downloaded from the database), followed by the mitochondrial genes used in the alignment (*Cyb*, *CO1*, *12S* and *16S*). Complete mitogenomes are written in bold. The geographic groups are highlighted by different colours: green for *F. pyrropus* from Central Africa; red for *F. pyrropus* from West Africa; and blue for *F. anerythrus* from Central Africa.
Abbreviations: CBGP: *Centre de Biologie pour la Gestion des Populations* - Small mammal Collection (France, Montferrier-sur-Lez); FMNH: Field Museum of Natural History (Chicago, USA); MNHN: *Muséum national d'Histoire naturelle* (Paris, France); MVZ: Museum of Vertebrate Zoology (Berkeley, USA); RMCA: Royal Museum for Central Africa (Tervuren, Belgium); ZFMK: Zoological Research Museum A. Koenig (Bonn, Germany). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The ecological niches were reconstructed using variables available in the WorldClim 2.1 [29] and ENVIREM [30] datasets at 2.5 min resolution (approximately 4.5 × 4.5 km gridcell). For each dataset, the least correlated variables ($|r| < 0.7$) in a 300 km radius around the occurrence records were selected using the caret R package [31]. The ecological niche modelling was performed with the MaxEnt (Maximum Entropy) algorithm [32] with ENMTools in R [33] using 70 % of the dataset points as training. To account for the slight differences in results due to the use of a machine learning algorithm, the ecological niche modelling was repeated 10 times. The area under the curve (AUC) was used as the measure of model accuracy, with a value of 0.7 often cited as the threshold for acceptable accuracy and a value of 1 indicating perfect model fit [34,35].

The niche of *F. pyrropus* was inferred using 39 occurrence records (Appendix B), including data from 17 sequenced specimens, 12 examined museum specimens, and 10 photographed squirrels, which finally resulted in 33 occurrence records after spatial thinning.

The niche of *F. anerythrus* was inferred using 49 occurrence records (Appendix B), including data from 40 sequenced specimens, 4 examined museum specimens, the holotype, and 4 photographed squirrels, which finally resulted in 42 occurrence records after spatial thinning.

The niche of *F. leucostigma* was inferred using 50 occurrence records (Appendix B), including data from 14 sequenced specimens, 14 examined museum specimens, the syntype, and 21 photographed squirrels, which finally resulted in 45 occurrence records after spatial thinning.

The niche of MPXV was inferred using 225 occurrence records, including seven animal cases and 218 human index cases deduced from published epidemiological data (Appendix C), which resulted in 139 occurrence records after spatial thinning. To determine if the ecological niche of MPXV fits well with that of squirrels, niche overlap comparisons were performed with ENMTools in R [33] using Schoener's D [36] and Hellinger's I [37] metrics. The D and I metrics were obtained by comparing the estimated habitat suitability for each grid cell of the ecological niche models. The closer the D and I values are to 1, the more the niches overlap; in contrary, a value of 0 indicates no overlap between niches [37].

3. Results and discussion

3.1. Molecular phylogeny of Funisciurus

We sequenced the complete mitochondrial genome on a selection of 13 African squirrels of the tribe Protoxerini and aligned them with six outgroup species of the family Sciuridae. Our alignment of 19 complete mitochondrial genomes was used to reconstruct the maximum likelihood (ML) tree shown in Fig. 1a. The results strongly support the polyphyly of the species *F. pyrropus*: fire-footed rope squirrels from Central Africa (Cameroon and Burundi) form the sister-group of *Funisciurus leucogenys* (Waterhouse, 1842) (bootstrap percentage, BP = 100), whereas those from West Africa (Benin, Côte d'Ivoire, Liberia, Mali, and Togo) constitute the sister-group of *F. anerythrus* (BP = 100). To better interpret phenotypic traits in *Funisciurus* squirrels, particularly the pelage coloration, we also sequenced a mitochondrial fragment of the cytochrome *b* gene from 50 dried skin samples collected on RMCA and MNHN specimens. We also included all mitochondrial sequences available in GenBank for the genus *Funisciurus*. Based on the tree generated from this dataset of 95 specimens (Fig. 1b), we concluded that additional specimens of *F. pyrropus* collected in Benin, Côte d'Ivoire, Ghana, Liberia, and Guinea belong to the West African clade which is sister to *F. anerythrus*, whereas additional specimens of *F. pyrropus* collected in DRC, Gabon, and Burundi belong to the Central African clade which is sister to *F. leucogenys*.

3.2. Evidence for two species of fire-footed rope squirrels

The examination of museum specimens showed that the two groups of *F. pyrropus* have distinct pelage coloration: Central African specimens, including the holotype of *F. pyrropus,* have flanks with a contrasting dark grey/brown back and pure white belly (Fig. 2a,b), whereas West African specimens have a dark grey/brown back separated from the white underparts with a broad reddish/orange band which continues onto the fore and hind limbs (Fig. 2c). Both molecular and morphological data therefore agree to separate the squirrels previously identified as *F. pyrropus* into two species. The name *F. pyrropus* can be maintained for Central African specimens since the pelage pattern of the holotype (Fig. 2a, whose geographical origin in Bioko Island is doubtful [20]) is identical to that of MNHN specimens from Gabon (Fig. 2b), Central African Republic, Republic of Congo, and southern Cameroon (not shown). In agreement with the International Code of Zoological Nomenclature [38], the oldest available species name, *Funisciurus leucostigma* (Temminck, 1853), needs to be resurrected for specimens from West Africa since the pelage pattern of a mounted syntype from Ghana [39] is the same as that observed in MNHN specimens from Côte d'Ivoire (Fig. 2c) and Guinea (not shown).

3.3. Geographic distributions of rope squirrel species

Using key features of pelage coloration, we were able to confirm or re-identify the species name of several squirrels photographed and georeferenced in the iNaturalist database [27]. Our observations confirm that *F. leucostigma* is present in Côte d'Ivoire, Ghana, Guinea, and Mali, and suggest that it is also found in Burkina Faso and Sierra Leone. In addition, the pelage pattern of three squirrels identified as *F. anerythrus raptorum* photographed in Nigeria (*e.g.*, IDs 99,922,915, 257519781 and 258,407,719) is very similar to that of *F. leucostigma* (Fig. 2c), but clearly different from that of *F. anerythrus* specimens collected in Gabon (Fig. 2d) and southern Cameroon (not shown). The taxonomic status of rope squirrels from Benin and Nigeria has been controversial in previous classifications as they were included in either *F. anerythrus* or *F. pyrropus*, or considered as a possible distinct species [20,21,40]. Our observations therefore suggest that they belong to *F. leucostigma*, which means that *F. anerythrus* does not occur in West Africa.

The geographic distributions of rope squirrel species (*F. anerythrus*, *F. leucostigma*, and *F. pyrropus*) were further explored using an ecological niche modelling (ENM) method based on georeferenced occurrence records deduced from our molecular analyses and our observations of coat coloration in museum specimens and live squirrels photographed in

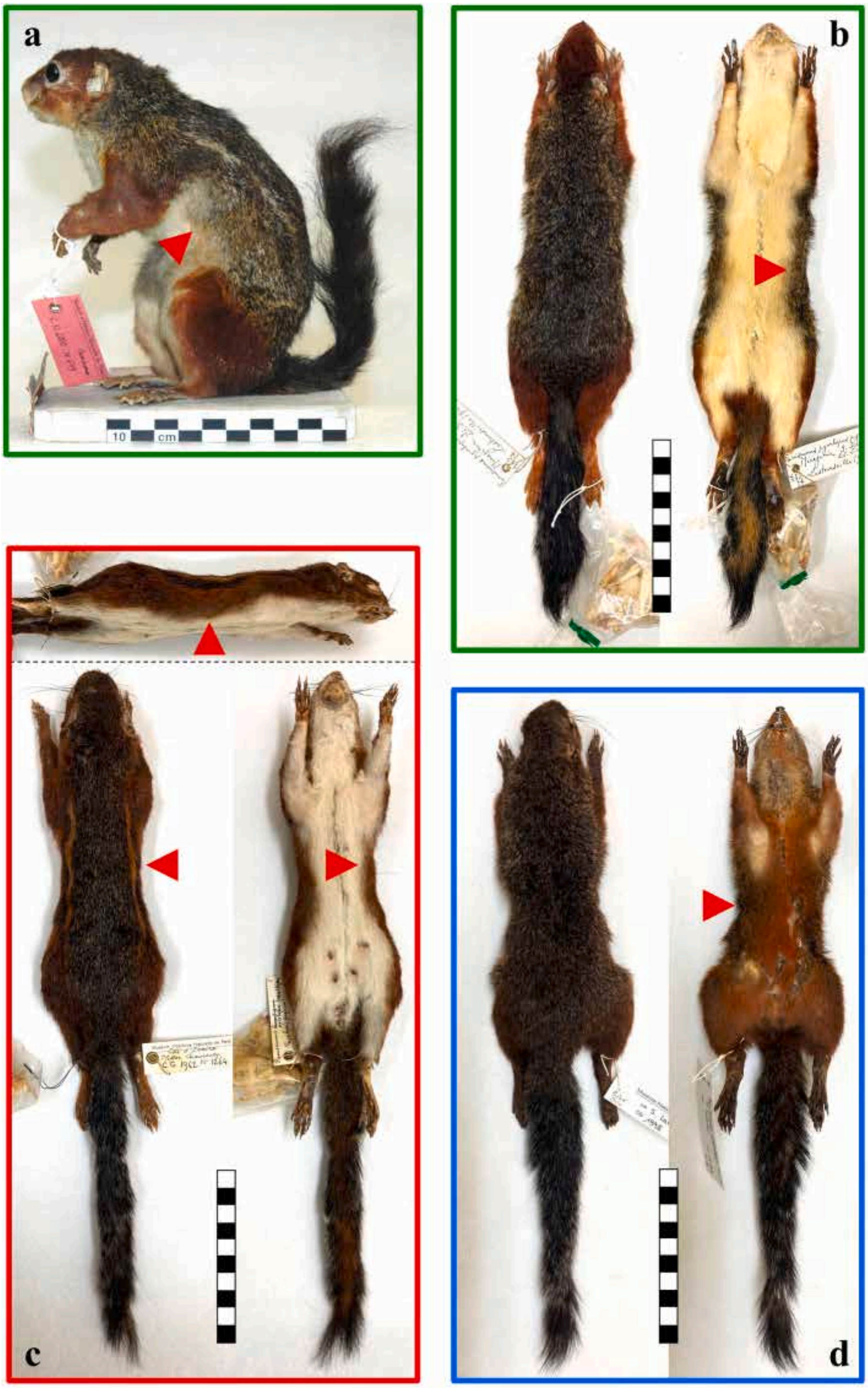

Fig. 2. Museum specimens of *Funisciurus pyrropus* (a, b), *Funisciurus leucostigma* (c) and *Funisciurus anerythrus* (d).
(a) Holotype of *F. pyrropus* (MNHN-ZM-MO-2000-619); the animal was a pet described in the island of Bioko in the Gulf of Guinea. However, it was probably captured in mainland Equatorial Guinea or Gabon [20]; (b) specimen of *F. pyrropus* from Gabon (MNHN-ZM-MO-1959-137); (c) specimen of *F. leucostigma* from Côte d'Ivoire (MNHN-ZM-MO-1962-1264); (d) specimen of *F. anerythrus* from Gabon (MNHN-ZM-MO-1998-2094). The red arrows show the diagnostic differences in pelage pattern between the three species. Photos: Laura Flamme (2014) for the mounted holotype of *F. pyrropus*; AH for others. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

African forests (iNaturalist database [27]). The ecological niche inferred for *F. pyrropus* shows that the highest probabilities of occurrence ($\geq$ 0.75, in yellowish green to yellow in Fig. 3a) are found in two main blocks in Central Africa: the first one includes the rainforests of southern Cameroon, Equatorial Guinea, Gabon and western Republic of Congo while the second one covers the rainforests of eastern DRC and extends to Burundi, Rwanda, and Uganda. In the ecological niche of *F. leucostigma* (Fig. 3b), the best probabilities of occurrence form a continuous block in West Africa connecting the coastal regions of UGF and LGF (from Guinea in the west, through Liberia, Sierra Leone, Côte d'Ivoire, Ghana, Togo, and Benin to Nigeria and western Cameroon in the east). A very similar ecological niche was found when the iNaturalist

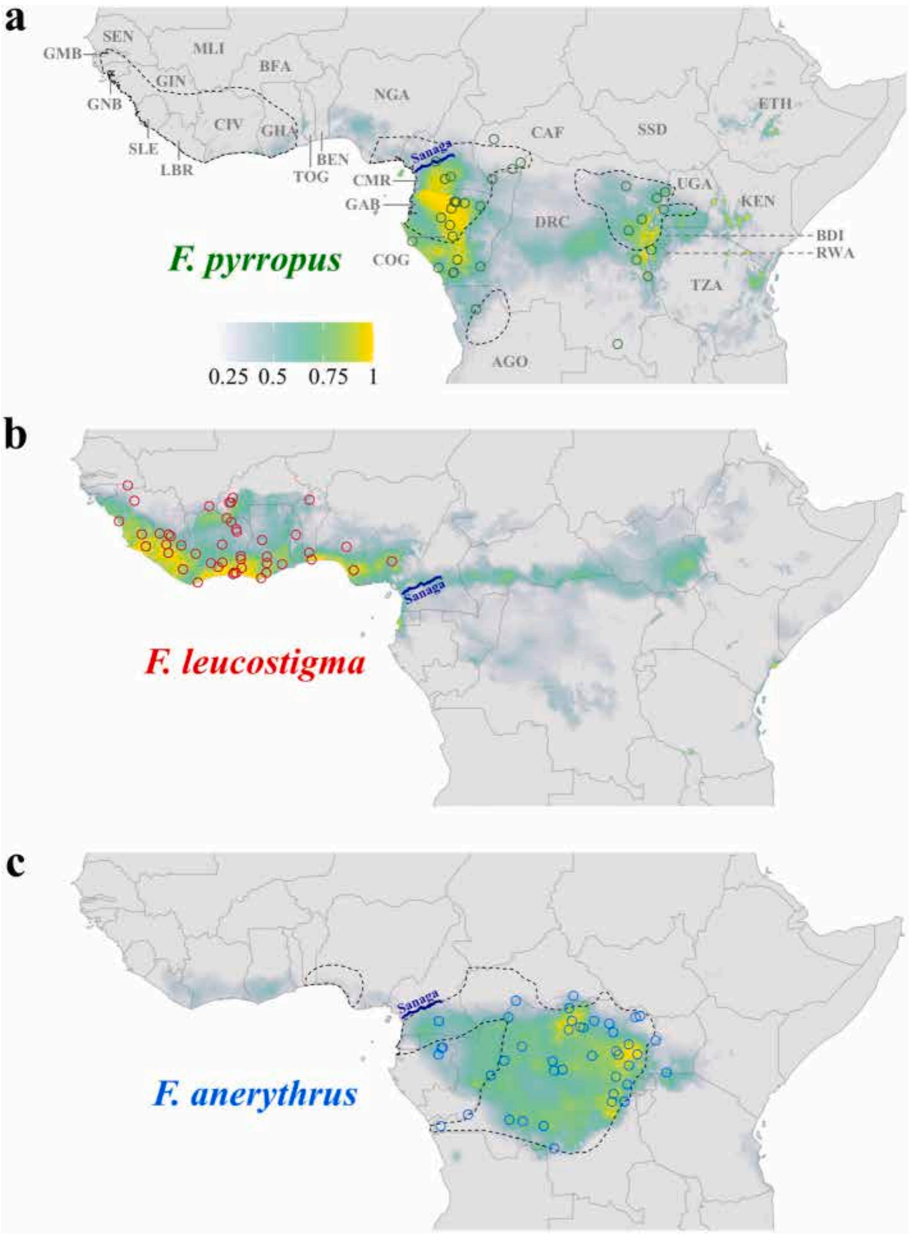

Fig. 3. Ecological niches of *Funisciurus pyrropus* (a), *Funisciurus leucostigma* (b), *Funisciurus anerythrus* (c).
Circles indicate reliable geographic localities used for ecological niche modelling. The ecological niches of *F. pyrropus*, *F. leucostigma*, and *F. anerythrus* were predicted using 33, 42, and 45 occurrence records respectively (AUC = 0.917, 0.948, and 0.949, respectively).
The probabilities of occurrence (p) are highlighted using different colours: blue grey for $p < 0.5$; turquoise green for $0.5 < p < 0.75$; yellowish green for $0.75 < p < 0.9$; and yellow for $p > 0.9$. The dashed line is the IUCN distribution of the species (only available for *F. pyrropus* and *F. anerythrus*) [21]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

occurrence records from Nigeria identified as *F. anerythrus raptorum* (see above) were removed (Appendix D). The ecological niche of *F. anerythrus* (Fig. 3c) is restricted to Central Africa where it covers the Congo Basin (DRC, northern Republic of Congo, northeastern Angola, southern Central African Republic, southern Cameroon and eastern Gabon) with some extensions in neighbouring countries, such as Uganda and Rwanda. The geographic distributions of the two sister-species identified as MPVX reservoirs in previous studies [10,11,14–19] are not overlapping: *F. anerythrus* is endemic to Central Africa, whereas *F. leucostigma* is endemic to West Africa. The barrier between the two species is the Sanaga River, the largest river in Cameroon which constitutes a significant obstacle to the dispersal of many species of arboreal mammals, including genets, primates and squirrels [11,20].

3.4. The two MPXV clades resulted from allopatric speciation in African rope squirrels

Our new systematic classification of rope squirrels allows us to better understand MPXV evolution. Indeed, previous studies have suggested that *F. anerythrus* is the main MPXV reservoir in Central Africa [10,11,14–18] and that *F. pyrropus* is a MPXV reservoir in Côte d'Ivoire [19]. Our analyses show that all *F. pyrropus* from West Africa, including those from Côte d'Ivoire actually belong to *F. leucostigma*, which we elevate here to full species status, and that this species is the sister to *F. anerythrus*. These results therefore imply that the squirrel recently identified as a MPXV reservoir in Côte d'Ivoire [19] belongs to *F. leucostigma*. In agreement with this point, the available photo of the animal [19] unambiguously shows that it exhibits the typical coat

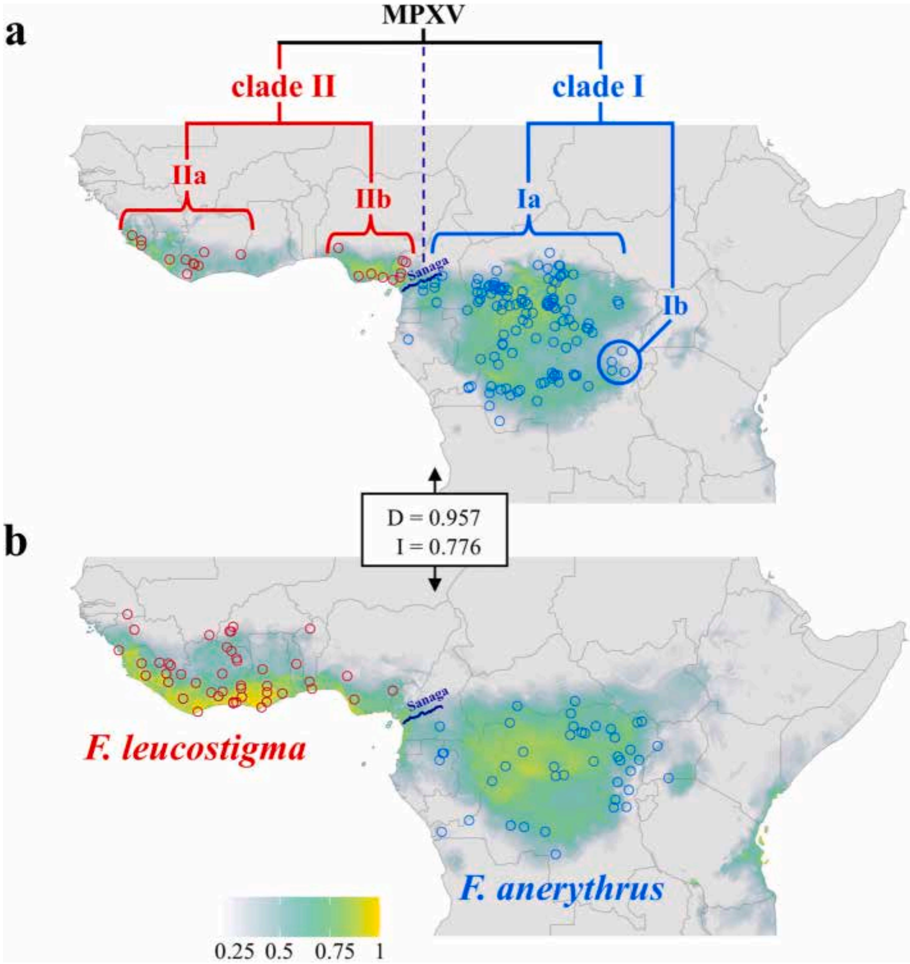

Fig. 4. Ecological niche of MPXV (a) compared to that inferred from occurrence records of both *Funisciurus anerythrus* and *Funisciurus leucostigma* (b).
Circles indicate reliable geographic localities used for ecological niche modelling. The probabilities of occurrence (p) are highlighted using different colours: blue grey for $p < 0.5$; turquoise green for $0.5 < p < 0.75$; yellowish green for $0.75 < p < 0.9$; and yellow for $p > 0.9$.
The ecological niche of MPXV (a) was predicted using 139 occurrence records with an.
AUC of 0.959. The four major clades (Ia, Ib, IIa, and IIb) found in the phylogeographic analysis of 164 MPXV genomes (full tree provided in Appendix E) are shown at the top.
The ecological niche shown in (b) was predicted using 87 occurrence records of both *F. anerythrus* and *F. leucostigma* with an AUC of 0.914.
Two overlap metrics were used to compare the ecological niche of MPXV with that of squirrels: Schoener's D [36] and Hellinger's I [37]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

coloration pattern observed in other *F. leucostigma* examined in this study, including specimens from Côte d'Ivoire, Ghana, and Guinea in the MNHN collection (Fig. 2c) as well as those photographed by iNaturalist explorers [27] in Côte d'Ivoire, Burkina Faso, Ghana, Guinea, and Sierra Leone.

Our phylogeographic analysis of MPXV genomes (Fig. 4a; Appendix E) corroborates previous studies [10,12,13] as we found maximal ML bootstrap percentages (BP =100) for a deep separation between two clades corresponding to Central Africa (clade I) and West Africa (clade II) and their subdivision into subclades Ia (covering most regions of Central Africa, including southern Cameroon [south of the Sanaga River], Gabon, Republic of Congo, southern Central African Republic, and DRC) and Ib (Kamituga region in eastern DRC) and subclades IIa (viruses from UGF countries, including Côte d'Ivoire, Ghana, Liberia, and Sierra Leone) and IIb (viruses from LGF countries, including Nigeria and Cameroon [north of the Sanaga River]).

The ecological niche of *F. anerythrus* (Fig. 3c) perfectly overlaps the geographic distribution of MPXV clade I in Central Africa [10,11] (Fig. 4a), while the ecological niche of *F. leucostigma* (Fig. 3b) fits well with the geographic distribution of MPXV clade II in West Africa [10,11] (Fig. 4a). In addition, the ecological niche of MPXV, inferred using animal cases and human index cases (Fig. 4a), overlaps the ecological niche built using all occurrence records available for *F. anerythrus* and *F. leucostigma* (Fig. 4b). The Schoener's and Hellinger's overlap metrics were found to be higher than the ones previously calculated between the ecological niches of MPXV and *F. anerythrus* [11]: $D = 0.957$ *versus* 0.931; $I = 0.776$ *versus* 0.746. In agreement with previous studies [10,11,13], our phylogeographic analysis of MPXV genomes confirms that the Sanaga River is an important biogeographic barrier between clades I and II. These two MPXV clades differ by genomic distances between 0.39 % and 0.44 %, whereas intra-clade distances do not exceed 0.20 %. Such a gap between inter- and intra-clade distances indicates that MPXV clades I and II have been isolated by the Sanaga River for a long time. Since the two squirrel sister-species are also separated by the Sanaga River, our results suggest that the two MPXV sister-clades evolve in allopatry in two sister-species of squirrels acting as reservoir hosts on either side of the Sanaga River, *F. anerythrus* in Central Africa [10,14–16] and *F. leucostigma* in West Africa [19]. Here, allopatry is explained by the fact that both *F. anerythrus* and *F. leucostigma* are not able to cross the Sanaga River. Consistent with that, the Sanaga River is the largest river in Cameroon, where it constitutes a natural barrier for the dispersal of several arboreal mammal species, including other squirrel species (*Funisciurus lemniscatus* [11,20] and *Funisciurus pyrropus*, see Fig. 2), small carnivores (genets), and several species of primates [11,20].

3.5. Limitations

Due to a strong bias toward epidemic studies, current data on MPXV clades I and II contain mainly human viruses. Only a few MPXV genomes were sequenced from animals, including several rope squirrels [10,16,19,41]. Since recent studies have revealed that MPXV genomes evolve much rapidly in humans than in animals [7,42], the divergence time between MPXV clades I and II cannot be reliably estimated with currently available data. We therefore consider that future studies should focus more on whole MPXV genomes directly sequenced from African squirrels, particularly in Cameroon, where both MPXV clades have been previously detected in humans on either side of the Sanaga River [13] and where the two *Funisciurus* species identified as reservoir hosts, *F. anerythrus* and *F. leucostigma*, can be sampled to fully characterize the ecological and environmental factors driving human transmissions.

CRediT authorship contribution statement

Manon Curaudeau: Writing – review & editing, Visualization, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Julian Kerbis Peterhans:** Writing – review & editing, Resources. **Thomas Le Flanchec:** Writing – review & editing, Formal analysis. **Emmanuel Gilissen:** Writing – review & editing, Resources. **Laurent Granjon:** Writing – review & editing, Resources. **Lison Barale:** Writing – review & editing, Data curation. **Antoine Gessain:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Alexandre Hassanin:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2025.101157.

Data availability

Raw reads used for assembling mitogenomes are available in NCBI Sequence Read Archive (Bioproject PRJNA1262415; Accessions: SAMN48453638-SAMN48453650). Annotated mitochondrial genomes are available in GenBank, accession numbers PV606393-PV606405. The DNA alignments are available in the Open Science Framework (OSF) platform at https://osf.io/m9ndq/.

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