JOURNAL OF GENERAL VIROLOGY

RESEARCH ARTICLE

Ozeki *et al.*, *Journal of General Virology* 2022;103:001796
DOI 10.1099/jgv.0.001796

MICROBIOLOGY SOCIETY

OPEN ACCESS

Identification of novel orthonairoviruses from rodents and shrews in Gabon, Central Africa

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Abstract

In Africa, several emerging zoonotic viruses have been transmitted from small mammals such as rodents and shrews to humans. Although no clinical cases of small mammal-borne viral diseases have been reported in Central Africa, potential zoonotic viruses have been identified in rodents in the region. Therefore, we hypothesized that there may be unrecognized zoonotic viruses circulating in small mammals in Central Africa. Here, we investigated viruses that have been maintained among wild small mammals in Gabon to understand their potential risks to humans. We identified novel orthonairoviruses in 24.6% of captured rodents and shrews from their kidney total RNA samples. Phylogenetic analysis revealed that the novel viruses, Lamusara virus (LMSV) and Lamgora virus, were closely related to Erve virus, which was previously identified in shrews of the genus *Crocidura* and has been suspected to cause neuropathogenic diseases in humans. Moreover, we show that the LMSV ovarian tumour domain protease, one of the virulence determination factors of orthonairoviruses, suppressed interferon signalling in human cells, suggesting the possible human pathogenicity of this virus. Taken together, our study demonstrates the presence of novel orthonairoviruses that may pose unrecognized risks of viral disease transmission in Gabon.

INTRODUCTION

Small mammals, including rodents and shrews, are hosts for highly pathogenic human viruses such as Lassa virus, Junin virus and orthohantaviruses, all of which belong to the order Bunyavirales [1]. Environmental destruction by deforestation, exploitation of the bush and immigration to undeveloped areas in sub-Saharan Africa may increase the likelihood of contact with wild small mammals, thus posing a risk of the emergence of novel viral diseases in the human population.

The genus *Orthonairovirus* is a member of the family *Nairoviridae*, belonging to the order Bunyavirales, which includes enveloped and negative-stranded RNA viruses [2]. Their genomes comprise large (L), medium (M) and small (S) segments that encode RNA-dependent RNA polymerase (L protein), glycoprotein precursor (GPC) and nucleocapsid protein (N protein), respectively [3]. In Africa, various orthonairoviruses, including Crimean–Congo haemorrhagic fever virus (CCHFV) and Nairobi sheep disease virus (NSDV), are associated with numerous diseases in both humans and domestic animals [4, 5]. Therefore, orthonairoviruses have been recognized not only as public health threats but also as micro-organisms of concern in the livestock industries in the region. Moreover, several orthonairoviruses have been identified in small mammals in

Received 30 April 2022; Accepted 02 September 2022; Published 10 October 2022
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Keywords: *Orthonairovirus*; small mammals; novel virus; Gabon; virus screening; surveillance.
Abbreviations: CCHFV, Crimean–Congo haemorrhagic fever virus; DDBJ, DNA Data Bank of Japan; DUGV, Dugbe virus; ERVEV, Erve virus; GPC, glycoprotein precursor; HAZV, Hazara virus; IFN, interferon; LMGV, Lamgora virus; LMSV, Lamusara virus; L protein, RNA-dependent RNA polymerase; N protein, nucleocapsid protein; NSDV, Nairobi sheep disease virus; OTU, viral ovarian tumour domain protease; TFAV, Thiafora virus.
The GenBank/EMBL/DDBJ accession numbers for the viral sequences are LC706368–LC706436 and LC671712–LC671787.
Two supplementary figures and two supplementary tables are available with the online version of this article.

Africa. Bandia virus and Thiafora virus (TFAV) have been identified in natal multimammate mice (*Mastomys* sp.) and musk shrews (*Crocidura* sp.), respectively, in Senegal, West Africa [6, 7]. Although their biological characteristics have yet to be elucidated, small mammal-borne orthonairoviruses may pose a potential risk of disease in humans.

Lassa fever is endemic in West Africa, where many clinical cases have been reported annually, and is thus recognized as a major public health concern [8]. Therefore, several investigations of small mammal-borne viruses have been conducted in the region, and multiple potential zoonotic viruses, including Old World mammarenaviruses, orthohantaviruses and orthonairoviruses, have been identified in rodents and shrews [7, 9–12]. In contrast, no clinical cases of Lassa fever have been reported in humans in Central Africa [8]. Nevertheless, some small mammal-borne viral surveillance studies have been conducted in this region and potential zoonotic mammarenaviruses, such as Bitu virus in Angola or lymphocytic choriomeningitis virus (LCMV) in Gabon, have been identified in rodents [13–16]. Furthermore, recent serological surveillance studies have provided evidence of past infection by small mammal-borne viruses, such as LCMV or orthohantaviruses, among residents of Central African countries, including Gabon [17, 18]. However, there are no reports on the identification of small mammal-borne orthohantaviruses or orthonairoviruses in the region, and the presence of bunyaviruses in shrews in Central Africa is largely unknown. Based on these perspectives, we hypothesized that unrecognized zoonotic viruses may be present in small mammals in Central Africa.

In this study, we surveyed the presence of Old World mammarenaviruses, orthohantaviruses and orthonairoviruses in wild small mammal populations in Gabon, Central Africa, to understand the potential risks of transmission of known and unknown zoonotic small mammal-borne viruses.

METHODS

Animal specimen collection

Rodents and shrews were captured between 2019 and 2020 using Sherman and Tomahawk traps in forests near suburban areas and bushes around human dwellings in Lambaréné, Central Gabon. After dissection, the harvested organ specimens were preserved at −80 °C until further analysis. Tissue RNA was extracted from the homogenate of the kidney, the organ where mammarenaviruses, orthohantaviruses and orthonairoviruses are highly prevalent, as described in previous viral surveillance studies [17, 19–21]. Briefly, kidney tissue was shredded with scissors and homogenized in phosphate-buffered saline (PBS). Tissue RNA was extracted from the supernatant of kidney homogenates using a PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Virus screening

Virus screening was performed using a PrimeScript II High Fidelity One Step RT-PCR kit (Takara Bio, Shiga, Japan). We initially targeted partially conserved nucleotide sequences of three viral genera, namely *Mammarenavirus, Orthohantavirus* and *Orthonairovirus*, all belonging to the order Bunyavirales. For reverse-transcription (RT) PCR detection of Old World mammarenaviruses and orthohantaviruses, we used previously described pan-viral family primer sets [11, 22]. For orthonairoviruses, a primer set was designed based on the alignment of the sequences of a highly conserved region in the L gene (Table S1 available in the online version of this article). RT-PCR was performed under the following conditions: 10 min at 45 °C, 2 min at 94 °C and 35 cycles of 10 s at 98 °C, 15 s at 45 °C and 10 s at 68 °C. After purification from the agarose gel using the QIAquick Gel Extraction kit (QIAGEN, Hilden, Germany), the PCR products were processed using the BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific) and analysed with an ABI 3500 capillary sequencer (Thermo Fisher Scientific) for Sanger sequencing. The obtained sequences were identified using BLAST searches (https://blast.ncbi.nlm.nih.gov). The sequences of the RT-PCR amplicons were deposited in the DNA Data Bank of Japan (DDBJ) database under the following accession numbers: LC706368–LC706436.

Animal species identification

The animal species captured in this study were identified by analysing their cytochrome *b* gene nucleotide sequences, as described previously [17, 22]. Briefly, tissue DNA was extracted from kidney homogenates using a DNeasy Blood and Tissue kit (Qiagen). PCR was performed using universal primer sets and PrimeSTAR GXL DNA Polymerase (Takara Bio) under the following conditions: 30 cycles each of 10 s at 98 °C, 15 s at 51 °C and 1 min at 68 °C. The animal species were identified using BLAST searches after performing Sanger sequencing.

Determination of the complete nucleotide sequences of viral genome

To determine complete viral genome sequences, we performed RT-PCR using RNA samples that were PCR-positive for the Lamusara virus (LMSV) and Lamgora virus (LMGV) genomes in the initial virus screening: 44 samples obtained from *Crocidura goliath* (virus prevalence of 44/128, 34.3%), a sample from *Lemniscomys striatus* (1/3, 33.3%), 6 samples from *Mus minutoides* (6/41, 14.6%), 2 samples from *Oenomys hypoxanthus* (2/6, 33.3%), 11 samples from *Praomys misonnei* (11/65, 16.9%) and 5 samples from *Rattus rattus* (5/16, 31.3%). To design the deduced primer sets, the nucleotide sequences of each genome segment

of Erve virus (ERVEV) and TFAV were aligned (GenBank accession numbers: ERVEV, JF911697–JF911699; TFAV, NC_039220-NC_039222). All the designed primer sets are listed in Table S1. Once the amplicons were confirmed to be LMSV or LMGV genomes, Sanger sequencing was performed repeatedly to close the sequence gaps. The terminal sequences of LMSV were determined using the SMARTer RACE 5′/3′ kit (Takara Bio) according to the manufacturer's instructions, and the RACE primers are listed in Table S1. All determined sequences of LMSV and LMGV were deposited in the DDBJ database under the following accession numbers: LC671712–LC671787.

Sequence data analysis

Identified nucleotide sequences of the LMSV and LMGV genomes were aligned with reference sequences of orthonairoviruses using MEGA 7 software with ClustalW [23, 24]. The nucleotide sequences of the orthonairoviruses were extracted from GenBank (accession numbers are listed in Table S2). Multiple sequence alignments were processed to construct phylogenetic trees using the maximum-likelihood method with 1000 ultrafast bootstraps using IQ-TREE [25]. Consensus phylogenetic trees were visualized and modified using FigTree version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Sequence similarity values were calculated using the *p*-distance matrix generated in MEGA7.

The open reading frames of the viral proteins were inferred and visualized using GENETYX software v13 (Genetyc, Tokyo, Japan). Signal peptides, transmembrane domains and *O*-glycosylation sites in the amino acid sequences of orthonairovirus GPCs were predicted using Signal IP, TMHMM and NetOglyc programs on the CBS server (http://www.cbs.dtu.dk/services/), respectively.

Recombination analysis was performed using Recombination Detection Program v5.5 with default settings [26]. The genome sequences of ERVEV and TFAV were used as reference sequences to which the newly identified sequences were aligned (GenBank accession numbers: ERVEV, JF911697–JF911699; TFAV, NC_039220-NC_039222). A potential recombination event was determined using at least four methods embedded in the program, and a *P*-value with Bonferroni correction <0.05 for multiple comparisons was applied as the threshold for significance.

Cells, plasmids and viruses

Human embryonic kidney (HEK293T) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). To construct the pC-CCHFV-OTU-HA and pC-LMSV-OTU-HA plasmids, the coding sequence of the CCHFV ovarian tumour domain protease (OTU) domain was amplified from the pCAG-Hyg-CCHFV L plasmid that constructed previously [27], and the coding sequence of the LMSV OTU domain (GenBank accession no. LC671712) was optimized for human codon usage and was synthesized (Azenta Life Sciences, South Plainfield, NJ, USA). Both cDNAs were inserted into mammalian expression vectors (pCXN2) fused with a C-terminal HA tag using an In-Fusion HD Cloning kit (Takara Bio). The vesicular stomatitis virus Indiana strain (VSV) stock was prepared using Madin–Darby bovine kidney cells maintained in modified Eagle's medium (Thermo Fisher Scientific) supplemented with 5% FBS and 1% P/S.

Plasmid transfection and induction of interferon signalling in human cells

The pC-CCHFV-OTU-HA and pC-LMSV-OTU-HA plasmids were transiently transfected into HEK293T cells using Trans-IT LT-1 (Takara Bio). At 30 h post-transfection, cells were inoculated with VSV at an m.o.i. of 0.01 for 30 min of absorption time. Fresh DMEM was then added to the monolayer after washing with PBS, and cells were further incubated at 37 °C, 5% CO_2. Total RNAs from mock and infected cells were extracted at 12 h post-infection using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Cell lysates were collected using sodium dodecyl sulfate (SDS) lysis buffer containing 1% NP-40, 50 mM Tris/HCl (pH 8.0), 62.5 mM EDTA and 0.4% sodium deoxycholate, and further analysed using SDS-PAGE and Western blotting.

Quantification of human *Ifn-β* mRNA expression levels

The mRNA expression levels of human interferon-beta (*Ifn-β*) were quantified as described previously [28]. Briefly, PCR primers targeting the mRNA sequence of human *Ifn-β* and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) housekeeping genes were used as listed in Table S1. After the degradation of genomic contaminants was conduted using Recombinant DNase I (Takara Bio), quantitative RT-PCR was performed using a One Step TB Green PrimeScript Plus RT-PCR kit (Takara Bio) and an ABI 7500 thermocycler (Thermo Fisher Scientific), under the following conditions: 5 min at 42 °C, 10 s at 95 °C and 35 cycles of 5 s at 95 °C and 30 s at 60 °C. The relative fold change in expression levels was determined using the ΔΔCt calculation method and visualized using R software v3.2.2 (https://www.r-project.org).

Western blotting

Cell lysate samples were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Amersham, Munich, Germany). After the membrane was blocked using 5% skim milk for 1 h at room temperature, the membrane was incubated with rabbit

Table 1. Species of captured small mammals and results of virus screening

Mammal species	Common name	*Mammarenavirus*	*Orthohantavirus*	*Orthonairovirus*
Crocidura goliath	Goliath shrew	0/128*	0/128	**44**/128
Crocidura poensis	Fraser's musk shrew	0/1	0/1	0/1
Hybomys univittatus	Peter's striped mouse	0/3	0/3	0/3
Hylomyscus sp.	African wood mouse	0/2	0/2	0/2
Lemniscomys striatus	Typical striped grass mouse	0/3	0/3	**1**/3
Lophuromys sp.	Brush-furred mouse	0/7	0/7	0/7
Mus minutoides	African pygmy mouse	0/41	0/41	**6**/41
Mus musculus	House mouse	0/3	0/3	0/3
Oenomys hypoxanthus	Common rufous-nosed rat	0/6	0/6	**2**/6
Praomys misonnei	Misonne's soft-furred mouse	0/65	0/65	**11**/65
Rattus rattus	Black rat	0/16	0/16	**5**/16
Stochomys longicaudatus	Target rat	0/6	0/6	0/6
		0/281	0/281	69/281

*No. of viral RNA-positive individuals/number of captured animals. Positive number is indicated in **boldface**.

monoclonal anti-HA antibody (QED Bioscience, San Diego, CA, USA) at a dilution of 1:2500. For the detection of β-actin, mouse anti-β-actin monoclonal antibody (Sigma, St Louis, MO, USA) was used at a dilution of 1:5000. Primary antibody binding was further detected using HRP-conjugated anti-rabbit IgG antibody (Promega, Madison, WI, USA) or HRP-conjugated anti-mouse IgG antibody (Sigma). The HRP-labelled proteins were visualized using ECL prime (GE Healthcare, Chicago, IL, USA) and a luminescent image analyser (LAS-3000; GE Healthcare) according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were conducted using R software v3.2.2. The prevalence of orthonairoviruses in animal species was assessed statistically as an independent number using the chi-squared test to calculate the *P*-value (*P*), odds ratios (OR) and 95% confidence intervals (CIs). $P<0.05$ was considered significant. The statistical significance of human *Ifn-β* mRNA expression levels was determined using one-way analysis of variance (ANOVA) with post-hoc Tukey's test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

RESULTS

Identification of novel orthonairovirus-like sequences in specimens from captured small mammals

We captured 152 rodents and 129 shrews in and around Lambaréné in Gabon, Central Africa, and screened them for the presence of small mammal-borne viruses. Virus screening did not detect mammarenavirus or orthohantavirus sequences in any of the samples, whereas novel orthonairovirus-like sequences were detected in 24.6% (69/281) of sampled animals (Table 1). BLAST searches revealed that the newly identified sequences showed high similarity to the L gene of ERVEV or TFAV (62.8–74.4% identity), which were previously identified in *Crocidura* sp. in France and Senegal, respectively [29, 30]. Notably, the prevalence of viruses in *Crocidura* sp. (34.1%, 44/129 animals) was significantly higher than that in all captured rodents (16.4%, 25/152 animals; OR: 2.63, 95% CI: 1.50–4.62, $P<0.001$). Moreover, sequence similarity values among the identified sequences varied from 71.4–100%, and phylogenetic analysis of the RT-PCR amplicons indicated at least two distinct phylogenetic clusters; these viruses were therefore tentatively named after the location and hosts in which they were identified: Lamusara virus (LMSV) [place: Lambaréné; virus hosts: 'musaraigne' ('shrew' in French) and 'ra' ('rodent' in French)] and Lamgora virus (LMGV) [place: Lambaréné; virus hosts: '*C. goliath*' (the shrew species) and 'ra')] (Fig. S1).

Whole-genome analysis of novel orthonairoviruses

The complete genome sequence of LMSV, consisting of three segments, L (11741 nt), M (3961 nt) and S (2247 nt), was successfully determined in accordance with orthonairovirus genome organization. Each genome segment of LMSV (L, M, and S) comprised a single open reading frame encoding the L, GPC and N proteins, respectively (Table 2). Moreover, the terminal sequences of the LMSV genome formed complementary termini; the 5′-termini of all three segments started with

Table 2. Lengths of nucleotide and amino acid sequences of orthonairovirus open reading frames, including Lamusara virus and Lamgora virus

Viral proteins	LMSV	LMGV	ERVEV	TFAV	CCHFV	HAZV	NSDV	DUGV
L protein	11580 nt	ND	11592 nt	11586 nt	11838 nt	11772 nt	11976 nt	12111 nt
	3859 aa		3863 aa	3862 aa	3945 aa	3923 aa	3991 aa	4036 aa
GPC	3819 nt	3819 nt	3891 nt	3846 nt	5055 nt	4266 nt	4875 nt	4656 nt
	1272 aa	1272 aa	1296 aa	1281 aa	1684 aa	1421 aa	1624 aa	1551 aa
N protein	2016 nt	1860 nt	1893 nt	2022 nt	1449 nt	1458 nt	1449 nt	1452 nt
	671 aa	619 aa	630 aa	673 aa	482 aa	485 aa	482 aa	483 aa

ND, not determined.
CCHFV, Crimean–Congo haemorrhagic fever virus; DUGV, Dugbe virus; ERVEV, Erve virus; HAZV, Hazara virus; LMGV, Lamgora virus; LMSV, Lamusara virus; NSDV, Nairobi sheep disease virus; TFAV, Thiafora virus.

the sequence UCUCAAAGA, whereas the 3′-termini of all three segments ended with the sequence UCUUUGAGA, both of which are conserved within orthonairoviruses (Fig. S2). Unfortunately, we failed to recover the complete nucleotide sequence of the three LMGV segments, including their terminal sequences; however, the open reading frames of the partial L protein (4557 nt of the partial L gene), complete GPC and N protein were successfully determined. Finally, the complete nucleotide sequences of LMSV were recovered from the 23 samples of *C. goliath*, a sample of *P. misonnei* and a sample of *M. minutoides*, and the partial sequences of LMGV were recovered from two samples of *C. goliath*.

Phylogenetic analysis was performed for open reading frames within the genomes of orthonairoviruses, which indicated that the nucleotide sequences of all three segments of LMSV and LMGV were phylogenetically close to those of ERVEV and TFAV (Fig. 1a–c). This result was consistent with the results of partial L gene sequences amplified by RT-PCR. Moreover, pairwise comparisons of nucleotide and amino acid sequences revealed that LMSV and LMGV presented higher sequence similarities to ERVEV and TFAV compared with other orthonairoviruses (Table 3). In particular, both LMSV and LMGV showed the highest sequence similarity values for the L and N genes with TFAV. Furthermore, LMSV and LMGV mutually

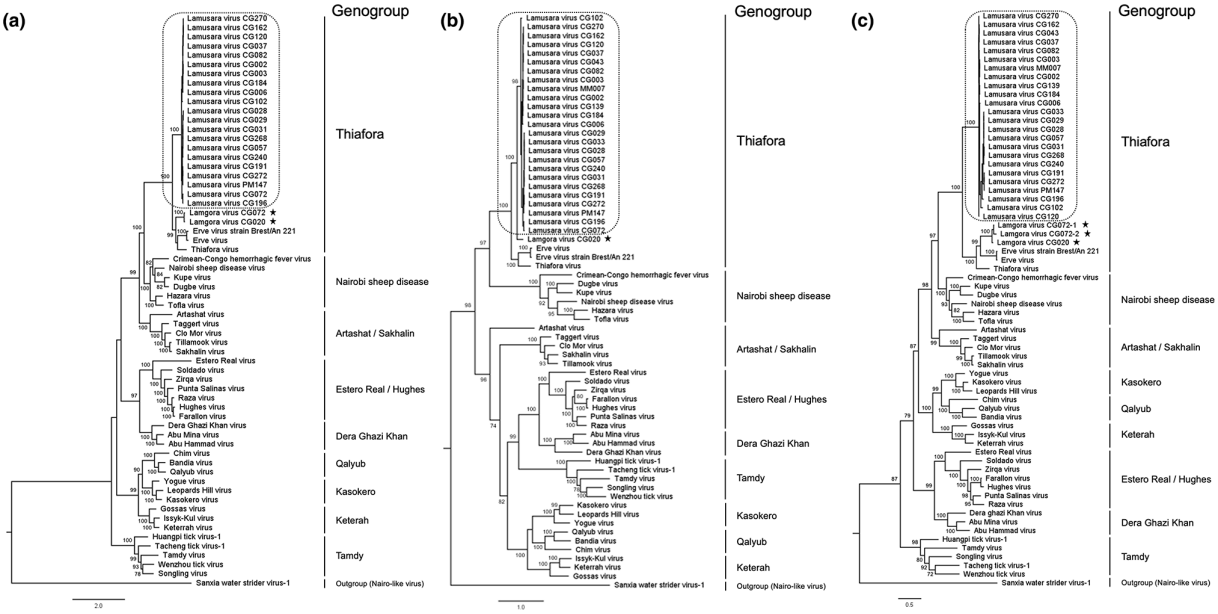

Fig. 1. Phylogenetic analysis of orthonairoviruses, including novel Lamusara virus (LMSV) and Lamgora virus (LMGV). Maximum-likelihood phylogenetic trees of LMSV and LMGV were constructed based on L protein partial coding sequences [at positions 6913–11 471 nt on LMSV L segment (DDBJ accession number: LC671712)] (a), GPC complete coding sequences (b) and N protein complete nucleotide sequences (c) using IQ-tree with 1000 bootstraps [25]. Bootstrap values of ≧70% are shown at the nodes of the trees. Nine genogroups of the genus *Orthonairovirus* are shown on the right side of the trees [29]. The LMSV clusters in each segment are circled by a broken line and the LMGV strains are marked by black stars. The scale bar indicates nucleotide substitutions per site.

Table 3. Pairwise comparisons of nucleotide and amino acid sequences among representative orthonairoviruses

	Nucleotide (%)							
	LMSV	**LMGV**	**ERVEV**	**TFAV**	**CCHFV**	**HAZV**	**NSDV**	**DUGV**
L								
LMSV		**68.6**	65.0	66.8	53.5	52.0	52.7	54.1
LMGV	66.9		67.1	**68.4**	53.0	53.1	53.3	55.3
ERVEV	69.6	67.9		68.8	52.4	51.3	51.5	53.1
TFAV	**72.0**	**69.0**	73.9		52.2	51.2	52.3	52.6
CCHFV	47.7	45.6	47.9	49.1		63.4	64.5	62.9
HAZV	48.7	47.4	49.7	49.9	66.8		65.4	63.8
NSDV	48.1	46.7	48.9	50.8	67.5	71.2		66.3
DUGV	48.7	47.2	48.7	49.8	65.2	67.8	70.2	
GPC								
LMSV		**82.5**	67.9	69.0	43.8	44.8	43.5	45.3
LMGV	**92.6**		66.7	67.7	43.6	44.6	43.4	45.1
ERVEV	68.5	68.3		70.0	43.5	44.9	45.0	45.1
TFAV	70.8	70.4	73.0		42.9	44.2	43.1	44.7
CCHFV	35.1	35.4	37.4	35.6		49.7	53.6	53.4
HAZV	39.5	39.2	39.7	39.5	41.6		59.1	54.7
NSDV	37.2	37.2	37.6	37.2	45.8	44.4		58.8
DUGV	37.0	36.9	37.2	35.9	44.1	50.4	55.6	
N								
LMSV		59.9	60.9	**62.3**	53.3	52.9	54.7	40.6
LMGV	61.4		68.9	**70.9**	52.4	51.2	52.3	51.4
ERVEV	61.2	72.9		67.9	51.7	51.2	51.5	51.4
TFAV	**62.5**	**76.2**	60.8		52.6	50.2	51.7	51.7
CCHFV	45.5	42.9	42.2	44.9		61.1	63.1	60.4
HAZV	44.7	41.6	42.1	42.7	60.3		63.2	59.9
NSDV	46.0	43.1	43.8	45.3	62.2	64.0		63.5
DUGV	41.8	42.3	44.2	44.2	57.7	56.2	60.5	
	Amino acid (%)							

Partial sequence of L protein on LMSV L-segment (at position 6,913–11,471 nt on DDBJ accession number LC671712), and complete nucleotide and amino acid sequences of GPC and N protein were analysed using MEGA 7 software (https://www.megasoftware.net/). Percentage values of nucleotide and amino acid sequence identities are indicated in the upper right half and lower left half, respectively. Sequence identity values between LMSV and LMGV are highlighted in grey. Highest nucleotide and amino acid identity values are shown in **boldface**. LMSV, Lamusara virus [DDBJ accession numbers: LC671712–LC671714 (CG002)]; LMGV, Lamugora virus [DDBJ: LC671780–LC671782 (CG020)]; ERVEV, Erve virus; TFAV, Thiafora virus; CCHFV, Crimean–Congo haemorrhagic fever virus; HAZV, Hazara virus; NSDV, Nairobi sheep disease virus; DUGV, Dugbe virus. GenBank accession numbers are listed in Table S2.

showed the highest sequence similarity for the GPC genes. According to criteria described by Walker *et al.*, both LMSV and LMGV should be grouped into the Thiafora genogroup in the genus *Orthonairovirus*, based on the amino acid sequence similarity values (>52%) of the N gene among LMSV, LMGV, ERVEV and TFAV [29].

Our phylogenetic analysis also demonstrated that all three encoded proteins of LMSV and LMGV formed unique phylogenetic clusters that differed from those of the other orthonairoviruses (Fig. 1a–c). The constructed phylogenetic trees showed that there were several phylogenetic branches present in the LMSV clusters of each segment, whereas the nucleotide sequences were highly conserved among the LMSVs: >90.6% for L genes, >90.5% for GPC genes and >89.1% for N genes. Moreover, we found that the GPC gene sequences were highly conserved between LMSV and LMGV (82.5 and 92.6% at the nucleotide and amino acid levels, respectively), whereas the sequences of the L and N genes were relatively divergent (L: 68.6 and 66.9%

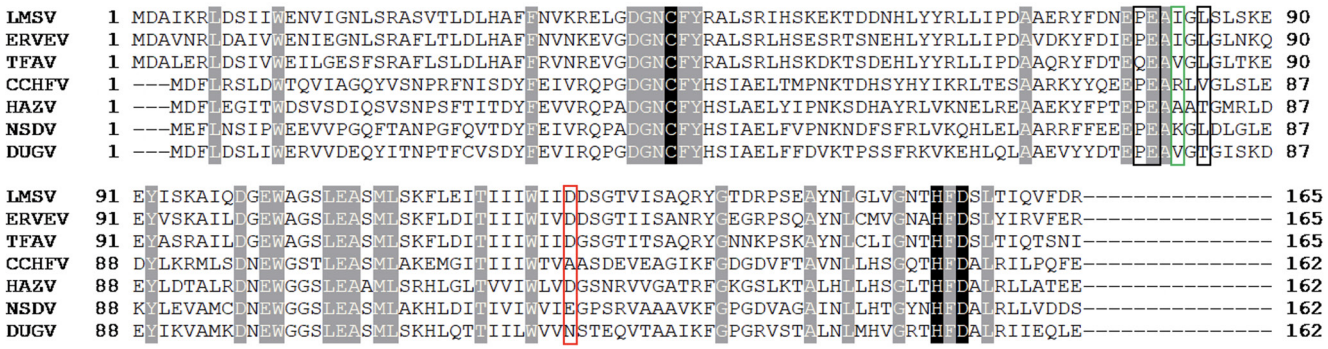

Fig. 2. Sequence comparison of the ovarian tumour domain proteases of orthonairoviruses. Sequence alignment of the ovarian tumour domain protease (OTU) was conducted based on the amino acid sequences of representative orthonairoviruses, including LMSV, Lamusara virus; ERVEV, Erve virus; TFAV, Thiafora virus; CCHFV, Crimean–Congo haemorrhagic fever virus; HAZV, Hazara virus; NSDV, Nairobi sheep disease virus; and DUGV, Dugbe virus. The catalytic triad residues are highlighted in black and the conserved residues are highlighted in grey. Putative residues influencing the deISGylation activity of all aligned orthonairovirus OTUs are indicated by a red box. Residues influencing the ISGylation activity of ERVEV OTU are indicated by black boxes. Residues influencing the species specificity of ERVEV OTU are indicated by a green box.

at the nucleotide and amino acid levels, respectively; N: 59.9 and 61.4% at the nucleotide and amino acid levels, respectively; Table 3). Intriguingly, the sequence length of the LMSV GPC gene was identical to that of the LMGV GPC gene (3819 nt/1272 aa), whereas the sequence lengths of the N genes differed considerably (LMSV: 2016 nt/671 aa, LMGV: 1860 nt/619 aa). Therefore, LMSV and LMGV were considered to be distinct; however, the differences in sequence similarities between L, GPC and N genes indicated that LMSV and LMGV may have experienced genetic reassortment events.

Genetic recombination is known to occur in orthonairoviruses, which enables them to acquire significant genetic diversity [4, 31, 32]. Therefore, we conducted recombination analysis to detect potential recombination events among the Thiafora genogroup orthonairoviruses. In our phylogenetic analysis, several phylogenetic branches were confirmed in the LMSV cluster; however, no potential recombination signals were detected for either the LMSV or LMGV (data not shown).

Molecular analysis of the LMSV ovarian tumour domain protease

The L protein of orthonairoviruses contains a viral OTU that functions as a virulence determinant of orthonairoviruses by suppressing the interferon (IFN)-mediated innate immune response [33]. To determine the possible pathogenicity of the novel viruses, we performed a sequence comparison analysis of the orthonairovirus OTUs. Although we were unable to identify the OTU sequence of LMGV, the amino acid sequences of the putative OTU region within the L protein of LMSV were identified and compared with those of other orthonairoviruses to predict the characteristics of the LMSV OTU (Fig. 2). The catalytic triad residues (C43, H154 and D156) in the LMSV OTU domain were highly conserved among orthonairoviruses. In particular, LMSV displayed greater amino acid sequence similarities to ERVEV (75.8%) and TFAV (74.5%) than to other orthonairoviruses (<43.2%). Notably, the OTUs of LMSV and ERVEV shared multiple amino acid residues that were previously predicted to be critical for deISGylation activity (P80, E81, L85 and D125) and species specificity (I83) [34, 35].

To examine whether LMSV OTU could suppress IFN signalling in human cells, we conducted VSV infection experiments in HEK293T cells over-expressing CCHFV-OTU-HA or LMSV-OTU-HA and confirmed IFN induction upon viral infection. The cells expressing CCHFV-OTU (3.77-fold increase to relative control) or LMSV-OTU (4.63-fold increase) significantly inhibited the upregulation of human *Ifn-β* mRNA expression levels compared with the empty vector-transfected cells (7.30-fold increase) (Fig. 3, upper panel). The expression levels of CCHFV-OTU and LMSV-OTU proteins in the transfected cells were similar (Fig. 3, lower panel). These results indicate that the LMSV OTU could interfere with IFN-mediated innate immunity in human cells.

Genetic characterization of the GPCs and N proteins of LMSV and LMGV

To understand the genetic characteristics of the novel viruses, we performed bioinformatic analysis of the protein-coding sequences of orthonairoviruses including LMSV and LMGV targeting their GPCs and N proteins.

The GPC of orthonairoviruses is a viral membrane protein that plays a key role in viral entry into host cells [3]. GPC comprises a signal peptide, a heavily *O*-glycosylated mucin-like domain, and two transmembrane structural proteins, Gn and Gc, which are proteolytically processed by a signal peptidase, SKI-I/S1P, and a furin protease [36–38]. Moreover, the GPC of CCHFV contains unique proteins, called GP38 and NSm, whose functions are to promote virus assembly and virion secretion [39].

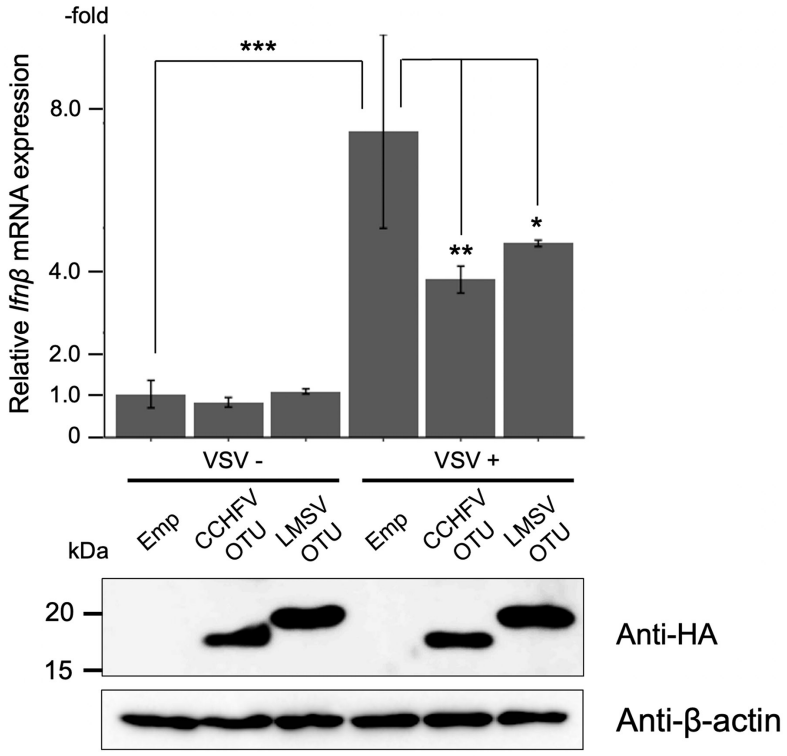

Fig. 3. Assessment of OTU activity in interferon-induced human cells. HEK293T cells were transfected with an empty vector or plasmids expressing CCHFV-OTU-HA or LMSV-OTU-HA, respectively. At 30 h post-transfection, cells were inoculated with VSV at an m.o.i. of 0.01. At 12 h post-infection, cellular RNAs were collected and human *Ifn-β* mRNA expression levels were assessed by quantitative RT-PCR using the ΔΔCt calculation method normalized with *Gapdh* mRNA expression levels. ΔΔCt values of empty vector-transfected mock cells were normalized to 1.0-fold and used as a relative control. The present data were obtained from a representative experiment performed in triplicate. Error bars represent standard deviation; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ (upper panel). Detection of over-expressed OTU proteins in mock- and VSV-infected cells. Anti-HA, HA-tagged OTU proteins; anti-*β*-actin, loading control (lower panel).

We constructed schematic diagrams of LMSV and LMGV GPCs (Fig. 4). Similar to orthonairovirus GPCs, both LMSV and LMGV GPCs contained a signal peptide, a mucin-like domain, Gn and Gc, and multiple proteolytic cleavage sites (RKLL^{273} and RKLL^{645}). No significant differences were identified in the organization of GPCs between LMSV and LMGV. Within the NSm protein of CCHFV, three transmembrane domains and two signal peptidase cleavage sites were predicted between Gn and Gc. Similar patterns were observed in the GPCs of Hazara and Dugbe viruses. In contrast, the NSm-like domain was not observed in the GPCs of LMSV and LMGV. Notably, the GPCs of LMSV and LMGV only possessed one transmembrane domain and one signal peptidase cleavage site between Gn and Gc; this structure was remarkably similar to the GPCs of ERVEV and TFAV.

The N protein of orthonairoviruses has DNA-specific endonuclease activity; however, its role in viral replication is unknown [40]. Alignment analysis of the amino acid sequences of N proteins revealed that five amino acid residues essential for endonuclease activity (R379, E382, K406, H446 and Q450 in LMSV) were highly conserved among orthonairoviruses (Fig. 5) [40, 41]. Moreover, long C-terminal extensions of N proteins, previously highlighted as unique features of ERVEV and TFAV, were observed in both LMSV and LMGV [29]. Taken together, these results demonstrate the close genetic relationship between LMSV, LMGV, ERVEV and TFAV, all of which are classified into the genogroup Thiafora of the genus *Orthonairovirus*.

DISCUSSION

In this study, we identified two novel orthonairoviruses, LMSV and LMGV, in small mammals captured in Gabon, Central Africa. Phylogenetic analysis demonstrated that LMSV and LMGV are closely related to ERVEV and TFAV (Fig. 1). Pairwise sequence comparisons further supported the close relationship between LMSV, LMGV, ERVEV and TFAV, all of which should be assigned to the Thiafora genogroup (Table 3). Furthermore, we propose that *Crocidura* sp. may be the main host for LMSV and LMGV

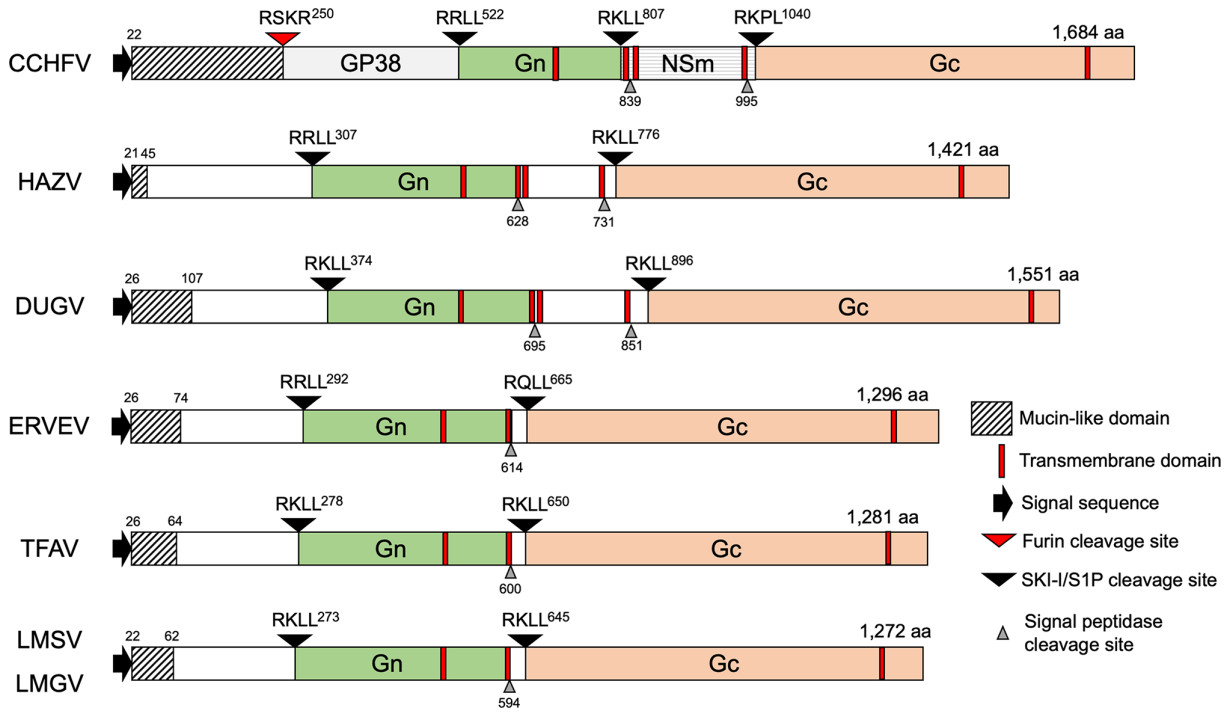

Fig. 4. Schematic diagram of deduced structures of the LMSV and LMGV glycoprotein precursors. Regions corresponding to a signal sequence (black arrow), mucin-like domain (oblique square), GP38 (grey), Gn (green), NSm (horizontal pattern) and Gc (orange) are indicated. The predicted signal peptidase, furin and SKI-I/S1P cleavage sites are indicated by triangles with the recognition sites and/or peptide sequences. CCHFV, Crimean–Congo haemorrhagic fever virus; HAZV, Hazara virus; DUGV, Dugbe virus; ERVEV, Erve virus; TFAV, Thiafora virus; LMSV, Lamusara virus; LMGV, Lamgora virus.

owing to the high prevalence of these viruses in shrews. Notably, ERVEV and TFAV were previously identified in *Crocidura* sp. in France and Senegal, respectively [29, 30]. These results suggest that LMSV and LMGV may share a common ancestor with ERVEV and TFAV, all of which may have originated from *Crocidura* sp.

LMSV and LMGV showed a high degree of sequence similarity in their GPC proteins, whereas L and N proteins were relatively distinct (Table 3). Together with our phylogenetic analysis, these results suggest that LMSV and LMGV have uniquely evolved from a common ancestor and have acquired significant sequence diversity. CCHFV is known to have the highest degree of sequence diversity among the arboviruses [4]. The genetic variation of CCHFV is supposed to be attributable to the accumulation of copying errors caused by the error-prone function of the L protein, enabling the virus to acquire a wide range of tick and vertebrate host adaptabilities. Moreover, genetic reassortment and recombination events are considered to contribute to the genetic diversity of orthonairoviruses [31, 32, 42]. In this study, we did not observe any evidence of a potential recombination event among novel viruses. However, our data indicate that reassortment events may have occurred within the Thiafora genogroup, since the LMSV and LMGV L and N proteins showed the highest sequence similarity with TFAV, whereas their GPCs were most similar to each other (Table 3). This outcome is, surprisingly, inconsistent with previous findings; the CCHFV M-segment displayed a higher frequency of genetic reassortment compared to the L- and S-segments, and showed the most variable degree of sequence diversity among the three segments [4, 31, 43]. For instance, the CCHFV ZT15-90 strain recently found in Zambia was described as a genetic reassortant: the M-segment originated from Asian CCHFV strains, whereas the S-segment originated from African CCHFV strains [44]. The mechanism by which the M-segment of CCHFV shows highly frequent reassortment patterns remains unknown. Taken together, further surveillance studies are needed to explore the depth of genetic variation in small mammal-borne orthonairoviruses circulating in wildlife in Gabon.

Most viruses in the genus *Orthonairovirus* are recognized as tick-borne viruses and have been isolated from various tick species, such as *Hyalomma* sp. for CCHFV or *Haemaphysalis* sp. for NSDV [3, 4]. It remains unclear whether LMSV and LMGV are transmitted by ticks, as we did not focus on ticks in this study. However, we identified the genome sequences of LMSV and LMGV in various rodent and shrew species (Table 1). Therefore, our results indicate the probable horizontal transmission of novel orthonairoviruses, suggesting that ticks may be involved in viral transmission between rodents and shrews in Gabon. Similarly, there are no reports that clearly demonstrate the transmission or isolation of ERVEV or TFAV from ticks, whereas antibodies against ERVEV were previously detected in wild mice captured in Germany, suggesting horizontal transmission of the

Fig. 5. Sequence comparison of the N proteins of orthonairoviruses. Sequence alignment of the N protein was conducted based on amino acid sequences of representative orthonairoviruses, including LMSV, Lamusara virus; LMGV, Lamgora virus; ERVEV, Erve virus; TFAV, Thiafora virus; CCHFV, Crimean–Congo haemorrhagic fever virus; HAZV, Hazara virus; NSDV, Nairobi sheep disease virus; and DUGV, Dugbe virus. Conserved residues are highlighted in grey and the residues responsible for endonuclease activity are highlighted in black.

virus among small mammals [45]. Further studies are required to understand the mode of transmission of LMSV and LMGV, particularly focusing on their association with tick migration from animal to animal.

We also demonstrated the close relationship between LMSV, LMGV and ERVEV through genetic comparison analysis of the deduced viral protein organization, including OTU, GPC and N proteins (Figs. 2, 4 and 5). In particular, the OTU of orthonairovirus is thought to be a virulence factor that suppresses IFN-mediated innate immune response [33]. Specifically, OTU has broad deubiquitinase activity, which is involved in deISGylation by cleaving the ubiquitin-like IFN-stimulated gene, *ISG15*, thereby impairing IFN signalling in host cells [46, 47]. Moreover, the OTUs of different orthonairoviruses exhibited species specificity for ISG15; for instance, the Dugbe virus OTU poorly cleaved human ISG15 but efficiently processed sheep and bovine ISG15s [34, 35]. A previous study suggested that ERVEV may cause neuropathogenic diseases, including thunderclap headaches, in humans [48]. In addition, intracerebrally ERVEV-inoculated young mice showed signs of neurological symptoms, including acute encephalitis and disseminated neuron necrosis [30]. Notably, the ERVEV OTU was previously shown to cleave human and mouse ISG15, but not northern shrew ISG15, which is a probable virus host [34, 35]. These studies suggest that the ERVEV OTU may contribute

to the establishment of diseases in infected hosts. Here, we showed that the OTUs of ERVEV and LMSV share multiple amino acid residues that were previously reported to be responsible for deISGylation activity and species specificity (Fig. 2). Moreover, molecular analysis of the LMSV OTU showed inhibitory activity against the upregulation of *Ifn-β* mRNA expression stimulated by VSV infection in human cells (Fig. 3). Although the pathogenicity of ERVEV and LMSV has not been elucidated adequately, these results suggest that, like ERVEV, LMSV may employ OTU function to avoid IFN-mediated innate immune responses and may cause diseases in humans through transmission from small mammals.

In conclusion, this study identified two novel orthonairoviruses, LMSV and LMGV, in small mammals in Gabon, Central Africa. LMSV and LMGV demonstrated notably identical genome organization of GPC proteins but distinct divergence in L and N proteins, suggesting the unique evolution of these orthonairoviruses during circulation in wildlife. Phylogenetic analysis revealed that LMSV and LMGV are closely related to ERVEV and TFAV, and all these viruses have been identified in *Crocidura* sp. Genetic characterization of viral proteins also supported the close relationship between these small mammal-borne orthonairoviruses, and molecular analysis of the LMSV OTU suggested the possible virulence of the novel virus. Further studies are required to understand the pathogenicity, tropism and mode of transmission of LMSV and LMGV. In Gabon, surveillance studies on the seroprevalence of rodent-borne viruses, such as mammarenavirus and orthohantavirus, have been conducted among local residents [17, 18]. However, there have been no reports specifically targeting the seroprevalence of orthonairoviruses. Our findings provide novel insights into small mammal-borne viruses that may pose a threat to public health in Africa.

Funding information
This work was supported by the Science and Technology Research Partnership for Sustainable Development (SATREPS) of the Japan International Cooperation Agency (JICA) and the Japan Agency for Medical Research and Development (AMED), JP20jm0110013 and JP20jm0210072, and KAKENHI from the Japan Society for the Promotion of Science (JSPS), JP17KK0170 and JP21K10415.

Acknowledgements
We thank Ms Miku Takano (Nagasaki University) and Ms Izumi Suzumori (Japan International Cooperation Agency, JICA) for management of logistics, material transportation and linguistic support. We are also grateful to all staff in the Department of Emerging Infectious Diseases of Nagasaki University, CERMEL, IRET and JICA for their support and encouragement. We would also like to thank Editage (www.editage.com) for English language editing.

Author contributions
Conceptualization, T.O., J.Y. Methodology, T.O., H.A., L.C.B., R.H., Y.K., J.Y. Validation, T.O. Formal analysis, T.O. Investigation, T.O., H.A., Y.U., C.N., E.F.A., G.W.E.E., L.B.M.K., B.C.B.B.N., R.M., P.M., B.K.M., F.L.M.N., G.N.O., M.J.V.M.M., Y.I., S.O., J.Y. Resources, T.O., H.A., Y.U., C.N., E.F.A., M.H., K.Y., Y.K., J.Y. Data curation, T.O., H.A. Writing – original draft preparation, T.O. Writing – review and editing, H.A., J.Y. Visualization, T.O. Supervision, B.L., J.Y. Project administration, H.A., Y.U., C.N., B.L., J.Y. Funding, H.A., Y.K., J.Y.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Approval for animal collection in Gabon was granted by the ethical committee of the Centre National de la Recherche Scientifique et Technologique (CENAREST), and a Material Transfer Agreement was established and approved by CENAREST in accordance with the Access and Benefit-Sharing guidelines. All animal specimen collection procedures were conducted in compliance with applicable institutional and national guidelines for the use and handling of animals.

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