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Research Article

Genomic epidemiology and evolutionary analysis of Lassa virus from small mammals suggest bidirectional viral movement across humans and animals

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Abstract

Lassa fever is a viral haemorrhagic fever that poses a persistent public health threat in several West African countries, particularly Nigeria. The scarcity of Lassa virus (LASV) sequences isolated from small mammal reservoirs limits our knowledge and understanding of LASV genomic diversity and transmission dynamics. To address this knowledge gap, we sampled 1189 small mammals, including mice, rats, and shrews, from two LASV-endemic states in southern Nigeria (Ondo and Ebonyi States) and tested them for the presence of LASV RNA using reverse transcription-quantitative polymerase chain reaction. Selected quantitative polymerase chain reaction-positive samples were subjected to whole genome sequencing and small mammal speciation through next-generation sequencing outputs. We recorded an overall polymerase chain reaction positivity rate of 61.6%, with rat species demonstrating the highest LASV prevalence. We also conducted a serosurvey of 269 small rodents using indirect Enzyme-Linked Immunosorbent Assay (ELISA) and obtained an overall anti-LASV seroprevalence of 45%. Using the Nextera XT metagenomic sequencing protocol, we produced 55 LASV partial ($n = 28$) and full-length genomes ($n = 27$) from small mammals sampled, all of which clustered within sublineage 2g. LASV sequences generated from this study suggest that LASV variation is mostly driven by location, as isolates from this study tend to cluster more closely with other isolates collected from within the same region, rather than by collection date or host. However, samples collected from Ebonyi State were more closely related

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to isolates collected in Ondo State than to isolates from Edo, despite a larger physical distance. Overall, the data from this study suggest free movement of the virus across states in Nigeria, among humans and various non-human taxa. The finding of LASV in additional small mammal hosts suggests that the virus reservoir is vast and may include many small mammals not well-characterized.

Keywords **Lassa virus genomes, small mammals, LASV IgM and IgG, LASV genomic diversity, Nigeria**

Introduction

Lassa fever (LF) is a viral haemorrhagic fever that continues to pose a persistent public health threat across West Africa, particularly in Nigeria (Siddle et al. 2018). Human infection typically occurs through contact with infected rodent urine or faeces (Safronetz et al. 2013), with documented cases of human-to-human transmission predominantly in healthcare settings (Fisher-Hoch et al. 1995, Dan-Nwafor et al. 2019). The widespread prevalence of rodent reservoirs, particularly in sub-Saharan Africa (SSA), makes individuals living in these regions vulnerable to LF (McCormick et al. 1987, Demby et al. 2001, Olayemi and Fichet-Calvet 2020, Leirs et al. 2023).

In Nigeria, LF prevalence varies by region and year. In 2018, the Nigeria Centre for Disease Control confirmed 633 cases with a 29.2% fatality rate. Some of the most affected states were Edo (44%), Ondo (25%) and Ebonyi (11%). In 2020, there were 1189 cases with a 20.5% fatality rate mainly in Ondo (35%), Edo (32%), Ebonyi (6.8%), and Taraba (4.9%) states (Grace et al. 2021). Oftentimes, rural areas record more cases, due to practices like consumption of rodents contributing to LASV transmission to humans (Ejembi et al. 2019). Additionally, *Mastomys* spp., the known natural animal reservoir for LASV, has demonstrated a preference for cereal crops, typically cultivated by rural subsistence farmers. However, a recent study reports that LF may be moving from rural to urban areas (Cadmus et al. 2023, Al–Mustapha et al. 2024).

Previously, reported outbreaks were gradual with 25, 109, and 143 cases reported in 2015, 2016, and 2017 respectively. The outbreaks were characterized by seasonal peaks during the dry season and concentrated in high-risk clustered areas often linked to human-rodent interactions and environmental factors (Grace et al. 2021, Cadmus et al. 2023, Davis et al. 2025). However, a significant increase in annual reported cases began with the 2018 LF outbreak, with 633 cases and 171 deaths confirmed. The increase was a result of improved diagnostics and heightened awareness in Nigeria compared to early efforts, which relied on generic symptoms that mirror common illnesses such as malaria. Cumulative case counts and overall temporal trends suggest that 2018 and 2019 cases appeared to be significantly different from previous years, with elevated suspected cases continuing throughout 2019 (Dhillon et al. 2018, Happi et al. 2019, Grace et al. 2021, Redding et al. 2021).

In the wake of the 2018 outbreak, phylogenetic analyses have been performed on over 200 LASV genomes. These analyses revealed increased zoonotic transmission from a genetically diverse viral population in the natural rodent reservoir (Siddle et al. 2018). Subsequent research reported an increased prevalence of LASV-positive rodents spanning a wide range of species (Happi et al. 2022).

Bowen et al. (2000) initially identified four lineages of the virus. However, subsequent studies revealed the existence of seven lineages in all. Among these, Lineages I, II, and III are circulating in Nigeria, whereas Lineage IV has been found circulating in Sierra Leone, Guinea, and Liberia (Ehichioya et al. 2011). Lineage V has been proposed, consisting of isolates collected in Mali and Cote d'Ivoire, while Lineage VI has been characterized in isolates from *Hylomyscus pamfi* in Nigeria (Manning et al. 2015, Olayemi et al. 2016, Karan et al. 2019). Lineage VII was reported by Whitmer et al. and it originated from Togo and Benin Republic (Whitmer et al. 2018). This lineage was reported to be related to Lineages II and I, which are circulating in Nigeria (Whitmer et al. 2018). Lineage II splits into distinct sublineages IIa and IIb, predominantly found in southern Nigeria, where most LF cases have occurred since 2018 (Oloniniyi et al. 2018, Siddle et al. 2018).

Four rodent species have previously been identified as LASV reservoirs. Of these rodents, two were classified as wild types (*H. pamfi* and *Mus baoulei*), and these supplement the well-known commensal reservoirs, *Mastomys natalensis* and *Mastomys erythroleucus* (Lecompte et al. 2006, Olayemi et al. 2016, Yadouleton et al. 2019). Additionally, several other small mammal species (*Lemniscomys striatus*, *Praomys daltoni*, *Mus minutoides*, *Praomys rostratus*, *Rattus rattus*, *Tatera* spp., and *Crocidura* spp.) were reported to possess LASV-specific antibodies and viral RNA, suggesting transmission of the pathogen among small mammals and the prospect of multiple small mammal species-to-human spillover (Fichet-Calvet et al. 2014, Olayemi et al. 2018, Happi et al. 2022, Vučak et al. 2022).

Historically, serological assays have been used to identify rodents previously exposed to LASV. However, they cannot confirm the presence of the virus. A few studies reported widely variable Lassa virus seropositivity (8%–82.3%) by IgG (Demby et al. 2001, Fichet-Calvet et al. 2014, Kayem et al. 2023, Tiamiyu et al. 2024, Mariën et al. 2025). Estimates of seroprevalence in the general population reported by Doohan et al. (2024) range widely from 2.6% to 58.2%. This marked heterogeneity likely reflects differences in study design, population characteristics, and geographic settings, and underscores substantial gaps in the available evidence base. Collectively, these findings highlight persistent uncertainty in accurately defining the burden, transmission dynamics, and population-level risk of LF (Safronetz et al. 2013, Kenmoe et al. 2020, Doohan et al. 2024). In contrast, genetic approaches are effective for real-time identification and detection of viral RNA in various hosts. Molecular assays have successfully identified LASV-infected rodents in SSA, including Mali (Safronetz et al. 2013), Guinea (Lecompte et al. 2006, Fichet-Calvet et al. 2007, Fichet-Calvet et al. 2014, Karan et al. 2019), Côte d'Ivoire (Kouadio et al. 2015), Sierra Leone (Leski et al. 2015), and Nigeria (Monath et al. 1974, Olayemi et al. 2016, 2018, Agbonlahor et al. 2017, Happi et al. 2022).

Despite numerous reports confirming the presence of LASV in rodents using molecular methods, only a few complete annotated LASV genome sequences from rodents are available in GenBank. Since rodents are the most reported natural reservoirs of LASV, unravelling the virus's genetic diversity within the small mammal populations and comparing it to sequences isolated from other animal species and humans is crucial for understanding of the virus evolution and

development of future countermeasures. This study aimed to address this gap by sampling small mammals from homes of individuals previously confirmed with LF, to investigate the genetic diversity of the virus, it's exposure dynamics, and associated humoral immune response.

Materials and methods

Ethical approval

The study received ethical approval from the Nigeria Health Research Ethics Committee (NHREC no:01/01/2007), the National Veterinary Research Institute, Jos, Nigeria (AEC/03/120/22), and the Animal Care and Use Review Office of the United States Army Medical Research and Development Command.

Study design and sampling location

For this study, small mammals were trapped between May 2021 and October 2022 in two LF-endemic states of Ondo and Ebonyi in Nigeria, as previously described (Happi et al. 2024). In Ondo State, sampling was carried out in four distinct settlements within the Owo Local Government area, specifically Ehinogbe, Ijebu-Owo, Isuada, and Iyere. In Ebonyi State, small-mammal sampling was done in seven distinct localities, to include Hausa Quarters, Iboko, Nkaliki, Nkwagu, Nwezenyi, Odunukwe, and Onu-Enyim.

Small-mammal trapping, identification, and sample collection

Small mammals were captured using Sherman live-capture traps (H.B. Sherman Traps, Tallahassee, FL, USA) placed in the houses of consented and previously confirmed LF-positive participants and five to six surrounding neighbouring households. Trapping was conducted for four consecutive nights each week, moving from one location to another within the study communities. This was done for 2 weeks each month and for a total of 15 months. The small mammals targeted were rats, mice, and shrews.

The trapped animals were sedated using chloroform. Captured small mammals were identified to the genus level based on their physical characteristics and biometric data as described by Cunningham and Moors (1996). Blood, tissue sections (including liver, kidney, spleen, embryo, and testis), urine, oral, and rectal swabs were obtained from each captured animal, when possible, using a sterile necropsy procedure. Blood from each animal was processed for plasma, while tissues, swaps, and urine samples were preserved in TRIzol reagent (Invitrogen, CA, USA) or DNA/RNA Shield (Zymo Research, CA, USA) for subsequent molecular analysis. The sampling site coordinates in each house were recorded for each trap. All samples were aliquoted in duplicate, and a cold chain was maintained at 2–8°C during transportation to the laboratory where they were stored at −20°C prior to analysis. All laboratory analyses were performed at the African Centre of Excellence for Genomics of Infectious Diseases, Redeemer's University, Ede, Osun, Nigeria.

LASV detection by real-time PCR

The QIAamp Viral RNA extraction kit (Qiagen, Hilden, Germany) was employed according to the manufacturer's instructions for nucleic acid extraction. Subsequently, 3 μl of the isolated RNA from each sample was utilized for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to detect LASV nucleic acid. The Superscript III Platinum SYBR Green one-step RT-qPCR kit (Life Technologies, CA, USA) and specific primers targeting the L gene of the virus (LASV-NG forward, 59-YAC AGG GTC YTC TGG WCG ACC-39; LASV-NG reverse, 39-RAT GAT GCA RCT TGA CCC AAG-59), as previously described by Wulff et al. 1975 and Happi et al. 2022 were used for the amplification. Two replicates of each sample type were analysed, and the mean cycle threshold (Ct) value of each sample was documented.

LASV antibody detection using ELISA

To assess rodents (mice and rats) exposure to LASV, the ReLASV® Pan-Lassa Nucleoprotein-specific IgG/IgM (NP-IgG/NP-IgM) and prefusion Glycoprotein-specific IgG/IgM (PfGP-IgG/PfGP-IgM) Enzyme-Linked Immunosorbent Assay (ELISA) Test Kits (Zalgen Labs, LLC) were utilized to carry out an indirect test, adhering to the manufacturer's instructions. The plasma samples from randomly selected rodents (rats and mice) from the two study sites were used. Each of the study areas was considered as a block, and randomization was done in a ratio of 3:1 for each rodent type in Ondo and Ebonyi states, respectively, with no eligibility criteria. Six calibrators obtained by a three-fold serial dilution were used for both rat and mouse sample runs. A 1:100 dilution of the naïve samples (obtained from laboratory-reared rodents in the USA and Nigeria), test samples, and negative control was prepared using the sample diluent, after which 100 μl of the preparations was dispensed onto the microwell plates. The Optical Density (OD) was calculated using the ELISA machine configuration. The mean blank value was subtracted from both 450 and 630 nM, and the delta OD values were calculated by subtracting the (630 nm—mean blank) values from the (450 nm—mean blank) values. For the IgG plates, the negative cutoff was determined to be twice the OD value of the negative control, while values four times the OD value of the negative control were set as the cutoff for IgM plates. Samples with values higher than the cut-offs defined above were recorded as positive.

Statistical analysis

Data were documented serially using unique animal identification numbers. They were analysed using Epi Info version 8.0 software (Centers for Disease Control and Prevention, Atlanta, GA) and SPSS for Windows (version 25.0, SPSS Inc., Chicago, IL). Plots were prepared to illustrate results using GraphPad Prism version 8.0 (GraphPad Software, San Diego, California, USA) and Microsoft Excel. Discrete variables were compared using a test of proportion by calculating chi-square with Yates' continuity correction. Cycle threshold (Ct) values, which were normally distributed continuous variables, were compared by Student's *t*-tests and/or analysis of variance, as applicable. Multiple comparisons of the Ct values were done using Tukey's HSD or Bonferroni Post Hoc Multiple Comparison approach. All significance tests were two-tailed, and values of $P < .05$ indicated statistical significance.

Library preparation and LASV metagenomic sequencing

Library preparation and metagenomic sequencing were performed on samples that were positive by LASV RT-qPCR with a cycle threshold ≤ 30. Libraries were constructed on extracted viral RNA using the Nextera XT library preparation kit (Matranga et al. 2016). Paired-end

sequencing was accomplished using an Illumina NextSeq 2000 P2-300 cycle cartridge on a NextSeq 2000 instrument (Illumina, San Diego, CA, USA).

Additionally, nine LASV-positive small mammal samples previously collected (August 2019 to February 2020) from the same location and processed the same way as described above were included for sequencing and phylogeographic analysis.

Small mammal species identification

Host speciation was performed on a subset of LASV-assembled metagenomic sequenced data. This was achieved by using Next Generation Sequencing (NGS) raw data from 50 samples with LASV reads and a contig length of >500 nucleotides to identify and confirm the small mammal species infected with the virus. Briefly, we passed the raw reads from the next-generation sequencer through a metagenomics pipeline; Microsoft's Premonition Metagenomics Pipeline (https://www.microsoft.com/en-us/research/project/project-premonition/). The pipeline mapped raw reads from the sequenced samples to the most closely related organisms in the database to carry out the species identification. Krona charts (Ondov et al. 2011) were generated to visualize the results.

Genomic assembly and phylogenetic analysis of LASV

We performed *de novo* assembly and reference-based refinement with the viral-ngs pipeline (Daniel et al. 2019). To determine the evolutionary relationships of the study's sequences to known LASV diversity, we collected all publicly available LASV sequences from the National Center for Biotechnology Information (NCBI) Virus database. We filtered the sequence dataset to remove (i) laboratory strains, (ii) duplicates, (iii) sequences without collection times, and (iv) sequences <500 nucleotides. Sequences were trimmed to their coding regions for the L ($N = 754$) and S ($N = 1\,185$) segments, respectively, in sense orientation. We independently aligned the L and S segments using MAFFT v7.505 and curated the alignment manually (Katoh and Standley 2013). We reconstructed phylogenetic trees, including our partial and complete sequences, using IQ-TREE2 v2.2 (Minh et al. 2020) under Model Finder Plus (Kalyaanamoorthy et al. 2017) and assessed bifurcation support by UFBoot2 (Hoang et al. 2018). Additionally, nine LASV-genomes from small mammal samples previously collected (August 2019 to February 2020) from the same location (Ondo and Ebonyi) were processed and sequenced the same way as described above, and were included for downstream genomics and phylogeographic analyses. A Chart summarizing the study design is presented in Supplementary Fig. S10.

As all sequences fell within sublineage 2g, we filtered out all sequences with more than 10% ambiguous nucleotides and sequences below 50% segment length. We also filtered out all sequences lacking state-level geographic metadata for this study's phylogeographic reconstructions.

The final datasets were $N = 336$ for the L segment (44 new sequences from this study) and $N = 420$ for the S segment (53 new sequences from this study). For both segments and including the nine previously assembled LASV sequences, we reconstructed time-scaled phylogenies for each dataset using BEAST 1.10.5 (Drummond and Rambaut 2007). We used the General Time Reversible (GTR) substitution model with a gamma-distributed rate variation among sites across all global datasets, selected by BIC after model testing in IQ-TREE. We used a relaxed normal clock, with an informative lognormal prior and a constant growth coalescent tree prior. For each of the datasets, we combined two independent Markov Chain Monte Carlo (MCMC) chains of 250 million states run with the BEAGLE computational library. Parameters and trees were sampled every 25 000 steps, with the first 20% of steps discarded as burn-in. Convergence and mixing of the MCMC chains were assessed in Tracer v1.7 (Rambaut et al. 2018), to ensure the effective sample sizes of all estimated parameters were >200. We performed an asymmetric discrete trait analysis in BEAST version 1.10.5 with geographic states aggregated on a state level to reconstruct the location-transition history across an empirical distribution of 1000 time-calibrated trees sampled from each of the posterior tree distributions estimated above. We used Bayesian stochastic search variable selection to infer non-zero migration rates and identify the statistically supported transition routes.

We included a Markov jump counting procedure to investigate the timing and origin of geographic transitions, or Markov jumps, across the full posterior to account for uncertainty in phylogeographic reconstruction associated with sparse sampling. We used the Tree Markov Jump History Analyzer from the pre-release version of BEAST 1.10.5 to obtain the Markov jumps and their timings from posterior tree distributions. We used Tree Annotator 1.10 to construct Maximum clade credibility trees for all datasets.

Results

Samples tested for Lassa virus detection using PCR

LASV infection was evaluated using PCR in 1189 small mammals captured from Ondo State ($n = 690$ from 222 households) and Ebonyi State ($n = 499$ from 135 households). Of the small mammals morphologically identified to the genus level, 562 (47.3%), 455 (38.3%), and 172 (14.5%) were mice, rats, and shrews, respectively (Table 1). In all, a total of 3606 samples [distributed as: liver ($n = 544$), spleen ($n = 1088$), kidney ($n = 1152$), testes ($n = 449$), embryo ($n = 125$), urine ($n = 57$), oral swabs ($n = 95$) and rectal swabs ($n = 96$)] were tested and reported in this study.

LASV positivity rate using PCR in small mammals trapped in Ebonyi and Ondo States

PCR testing of at least three samples per small mammal determined LASV positivity, with a positive animal result if one sample was positive. The overall positive rate was 61.6% (732/1189). Ondo State had higher LASV positivity than Ebonyi State [67.1% (463/690) vs53.9% (269/499); $P < .0001$]. Rats showed higher positivity than other small mammals [73.4% (334/455) in rat vs 55.2% (310/562) in mice and 51.2% (88/172) in shrews; $P < .0001$] (Fig. 1). In Ondo State, rats had the highest positivity [76.4% (294/385)], while in Ebonyi State, mice had the highest [57.7% (177/307); $P = .02$].

LASV PCR positivity rate among sample types

The small mammal samples analysed for LASV were divided into tissue ($n = 3358$), swab ($n = 191$), and urine ($n = 57$) categories. LASV positivity was 40%–49% in tissues, 23%–35% in swabs, and 17% in urine (Supplementary Table S1). Oral swabs showed low positivity, while liver, spleen, and kidney had higher rates (Supplementary Fig. S1). Taken together, mean Ct values of all tissues

Table 1 Distribution of small mammals evaluated for Lassa virus infection in Ondo and Ebonyi States.

State	Study site	Small mammal type			
		***Mice* (*n* = 562)**	***Rats* (*n* = 455)**	***Shrews* (*n* = 172)**	***Total* (*n* = 1189)**
Ondo	Ehinogbe	53	109	13	175
	Ijebu-Owo	72	79	17	168
	Isuada	71	100	10	181
	Iyere	59	97	10	166
	ALL	255	385	50	690
Ebonyi	Hausa Quarters	12	28	59	99
	Iboko	14	0	3	17
	Nkaliki	91	30	37	158
	Nkwagu	31	5	11	47
	Nwezenyi	119	2	5	128
	Odunukwe	12	3	4	19
	Onu Enyim	28	2	3	33
	ALL	307	70	122	499

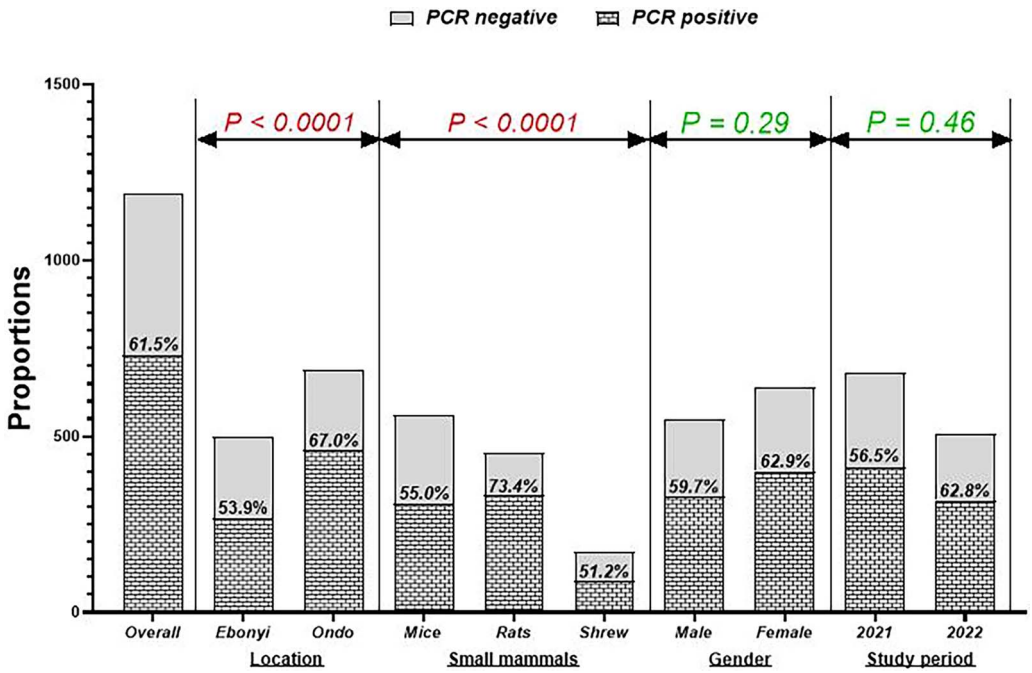

Figure 1 Prevalence of Lassa virus in small mammals based on location, species, gender and study period; the figure illustrates the sample sizes and LASV positivity across locations, small mammal types, gender as well as the study period; overall LASV positivity (61.5%) is also shown; statistical comparisons were performed by a test of proportion using the χ-squared test.

and swabs from Ondo State were lower than those from Ebonyi State (Supplementary Fig. S2). Additionally, mean Ct values were lower in the kidney, liver, and spleen of rats compared to shrews and mice (Supplementary Fig. S3).

LASV detection using ELISA in small rodents

Distribution of the immunological markers for LASV in small rodents

Of the 269 plasma samples (198 from Ondo State and 71 from Ebonyi State) evaluated for LASV antibody detection in rodents, an overall antibody positivity of 45.0% (121/269) was recorded. LASV antibody positivity sample rate was slightly, but not significantly, higher in Ebonyi [33/71 (46.5%)] compared to Ondo [88/198 (44.4%) [$P = .88$] (Table 2).

Complexity of the immunological response in LASV-exposed small rodents

Of the 121 LASV seropositive rodents, 51.2% had only one of the immunological markers positive (NP-IgG, NP-IgM, PfGP-IgG, and PfGP-IgM), while 48.8% had two or more of these markers. Combining IgM and IgG for each of the targeted proteins (NP and GP), the prevalence of LASV PfGP markers was significantly higher than NP markers [105/269 (39.0%) vs 52/269 (19.3%), respectively; $P = .0000008$]. However, detection of LASV IgM [105/269 (39.0%)] in small rodents was significantly higher compared with IgG [61/269 (22.7%); $P = .00004$].

Table 2 Lassa virus plasma antibody positivity from small rodents captured in Ondo and Ebonyi States.

Study area	Study sites	Total sample analysed	No. positive	% Seropositive
Owo (Ondo State)	Ehinogbe	2	2	100
	Ijebu-Owo	27	9	33.3
	Isuada	34	17	50.0
	Iyere	135	60	44.4
	ALL	198	88	44.4
Abakaliki (Ebonyi State)	Hausa Quarters	22	9	40.9
	Nkaliki	24	10	41.7
	Nwezenyi	17	8	47.1
	Onu-Enyim	8	6	75.0
	ALL	71	33	46.5

Comparison of the immunological response within the rodent types

The immunological responses of different rodent types in this study are shown in Fig. 2. Consistently, all rodents elicited a greater response to the glycoprotein compared to the nucleoproteins. The expressions were significantly higher in mice ($P = .000001$) than rats ($P = .04$). Similarly, overall response to IgM was substantially higher compared with IgG in both mice ($P = .005$) and rats ($P = .003$) (Fig. 2).

LASV genome sequencing assembly and phylogeny

We generated 55 partial ($n = 28$) and full-length ($n = 27$) genomes from small mammals sampled between May 2021 and October 2022 (47 rats and 8 mice). The complete sequences have been submitted to GenBank (accession numbers from PP431155 to PP431211). For analysis, we included nine previously assembled LASV genomes from small mammals (seven mice, one rat, and one shrew) also deposited in GenBank (accession number ON569369 to ON569379) in the sequenced genomes pool, as they have not been part of any previously published phylogenetic analysis. All new LASV sequences were generated from samples collected in Ondo State, except for three samples that were collected in Ebonyi in 2019 and 2020 (Supplementary Table S2). A map of the sampling areas for this study, as well as the Nigerian states from which existing sequences used in our analysis originated, is presented in Supplementary Fig. S4. We performed Bayesian phylogenetic reconstructions in BEAST by using the study's samples alongside all high-quality, full-length LASV S or L segment genomes available on GenBank, including both human and small mammal isolates.

In this study, LASV isolates were closely related to samples previously collected from other rodents, humans, and non-rodents across the region. Sequences tend to cluster more closely with other sequences collected from within the same region, as opposed to clustering by collection date or host. Our sequences from Owo in Ondo State were interspersed across 10 well-supported phylogenetic clusters and two singletons in the subtree annotated as sublineage 2g as per Ehichioya et al. (2019) in the S phylogeny (Fig. 3a). In the L phylogeny, our sequences clustered into five well-supported clusters and two singletons within sublineage 2g (Fig. 3b). Ancestral node probabilities and their bootstrap values for Fig. 3a and b are available in Supplementary Figs S5 and S6. We found that all our sequences ($N = 64$ for the S segment, $N = 52$ for the L segment) clustered within sublineage 2g, which is known to circulate in Edo, Ondo, Kogi, Delta, Anambra, and Ekiti States of Nigeria (Ehichioya et al. 2019) and for the first time in Ebonyi State. Notably, we found that our sequences from Abakaliki, Ebonyi State (2019–20) were more closely related to sublineage 2g sequences from Owo, Ondo State (2019–22).

All our phylogenetic clusters across both segments were closely related to sequences isolated from human cases in Owo and the wider Ondo State from 2017 to 2019. Based on the available metadata, the state with the highest amount of sampling that generated LASV genomes was Edo (62 genomes), followed by Ondo (58 genomes). Sampling peaked between 2016 and 2019, with the most genomes generated in 2018 (164) (Supplementary Fig. S7). We also found that a small cluster of sequences sampled from small mammals in Owo in 2021–22 was phylogenetically incongruent across well-supported clades in the L and S segments (Supplementary Fig. S8), suggesting possible reassortment owing to co-infections with different LASV lineages.

Our phylogeographic reconstruction suggests that the most recent common ancestor (MRCA) of the sublineage 2g S segment originated in Edo State, in late 1939 (95% Highest Posterior Density (HPD) 1929–51). The evolutionary rate for the S segment was estimated to be 7.8×10^{-4} nucleotide substitutions per site per year (95% HPD 6.7×10^{-4}–9.0×10^{-4}). The phylogeographic reconstruction suggests that the time to the most recent common ancestor (tMRCA) of the sublineage 2g L segment originated in Kogi State in the year 1940 (95% HPD 1929–51). Inconsistencies in resolving the ancestral state of sublineage 2g can be ascribed to sparse sampling. The evolutionary rate for the L segment was estimated to be 8.6×10^{-4} nucleotide substitutions per site per year (95% HPD 7.7×10^{-4}–9.6×10^{-4}). The L segment from one animal sample collected in Ondo State appears to have diverged from its closest sequenced relative 18 years before collection in 2021, suggesting some degree of isolation of the small mammal host, and therefore LASV from other populations. The group from which it diverged contains a virus isolated in Ondo State from 2015 to 2022, as well as from a goat in Ebonyi State in 2022 and a human sample from Edo State in 2018.

Amino acid substitutions of LASV study isolates compared to the ancestral strain

On the S segment, the highest prevalence of amino acid substitutions is found in the GP1 region of the Glycoprotein Precursor Complex (GPC), which contains the LASV receptor binding domain and may be subjected to higher selective pressure than the GP2 region, which contains transmembrane and intracellular domains (Fig. 4a; Supplementary Fig. S9) (Katz et al. 2022). A cluster of substitutions is present around the noncoding region between the NP and GPC Open Reading Frames (ORFs), including the region of the stable signal peptide, which helps to stabilize the spike complex (Katz et al. 2022).

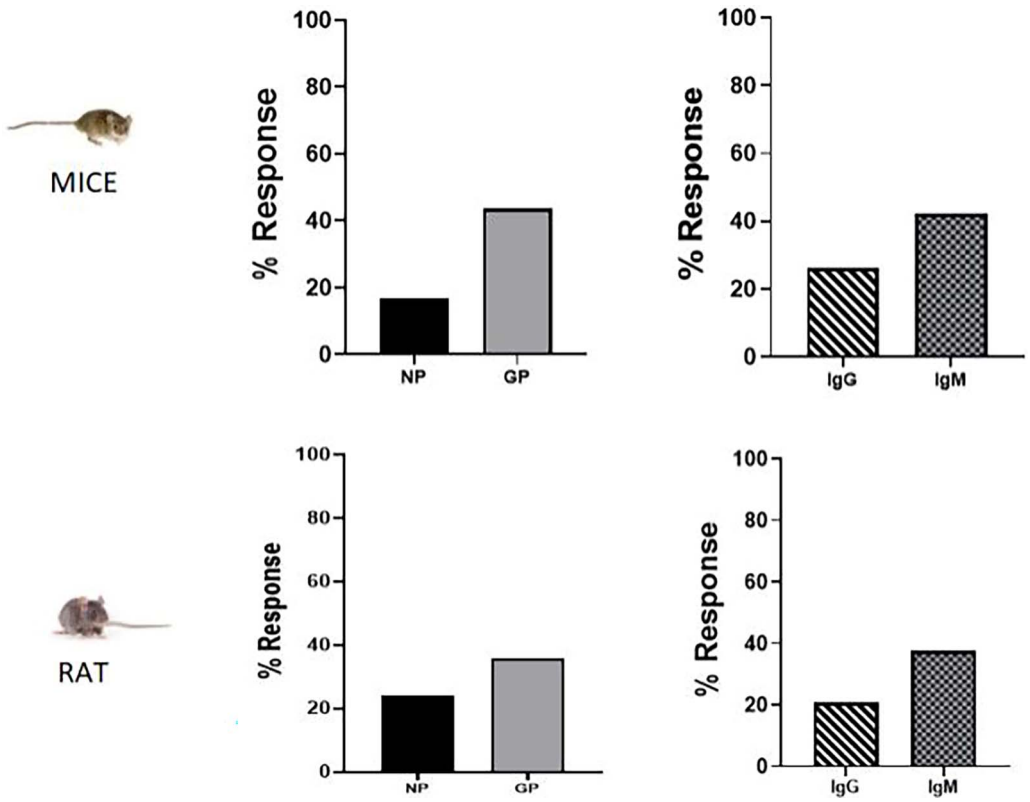

Figure 2 Response to immunological markers in LASV-infected rodents. NP (NP-IgG and NP-IgM), GP (PfGP-IgG and PfGP-IgM). IgG (NP-IgG and PfGP-IgG), and IgM (NP-IgM and PfGP-IgM) for rats and mice.

Similar to the S segment, most amino acid substitutions on the L segment clustered around the noncoding region between the L protein and Z protein ORFs (Fig. 4b). A moderate amount of amino acid substitution was present in the C-terminus of the Z protein in sublineage 2g, a region in which mutation has been associated with up to a 90% decrease in virus budding (Strecker et al. 2003). However, we cannot infer the functional effects of these substitutions without experimental characterization.

Movement of LASV across state boundaries

We identified several instances of LASV dispersal between states in our Bayesian phylogenetic reconstruction. Additionally, three sequences of sublineage 2g, which are closely related to several other 2g isolates collected in Ondo State, were collected for the first time in Ebonyi State.

For both segments, the highest number of interstate events occurred between Ondo and Edo States (Fig. 5a and b). There were significantly more introductions estimated from Ondo to Edo in the LASV S segment relative to the L segment (11.3 mean introductions [95% HPD 11.1–11.4] vs 6.8 introductions [95% HPD 6.7–6.9]; $P < .05$ by Wilcoxon rank-sum test). There was also a significantly higher mean number of cross-state events from Edo to Kogi and from Edo to Plateau for the S segment than for the L segment. Conversely, there was a significantly lower mean movement of the LASV S segment from Kogi to Edo than for the L segment. All our estimated geographic transitions are subject to sampling biases and phylogenetic uncertainty in the phylogeographic reconstructions. However, our reconstructions collectively support frequent directional dispersal from Ondo to Edo.

The year with the highest estimated number of interstate events for the LASV S segment was 2017 with 4.2 introductions, followed by 2015, 2018, and 2016 with 4.1, 3.4, and 3.3 introductions in each respective year (Fig. 5). For the L segment, the years with the most introductions were 2017, 2018, 2015, and 2016, with 3.7, 3., 6, 3.5, and 3.1 introductions, respectively.

Among our samples, there is more diversity in LASV sublineage 2g, maintained in Ondo State than in Ebonyi (Fig. 6). The clades defined by sequences collected around Owo, Ondo State, and the deepest nodes on the sublineage 2g S segment tree demonstrate a tMRCA between 0.1 and 12.4 years, while those of isolates from Ebonyi all have a tMRCA of <1 year.

GPS coordinates of sampling sites were recorded during collection and are plotted on magnified map areas of Ondo (A) or Ebonyi (B) States. Sampling locations are coloured based on tMRCA for the clade defined by each isolate and the deepest node on the sublineage 2g S segment tree, as calculated by Bayesian coalescent analysis. Map images were generated using OpenStreetMap, whose data is available under the Open Database License.

Small mammal species identification

The assembled metagenomic sequenced data were used to identify the sampled hosts based on genomic similarity to publicly available sequence databases (Fig. 7). Among the 50 randomly selected hosts, various small mammal species and mixed/crossed species were identified (Table 3). Twelve small mammal species with 92%–100% similarity to a reference species in the database were identified. The sampled host with 92%–100% identity were *Mastomys coucha* (4),

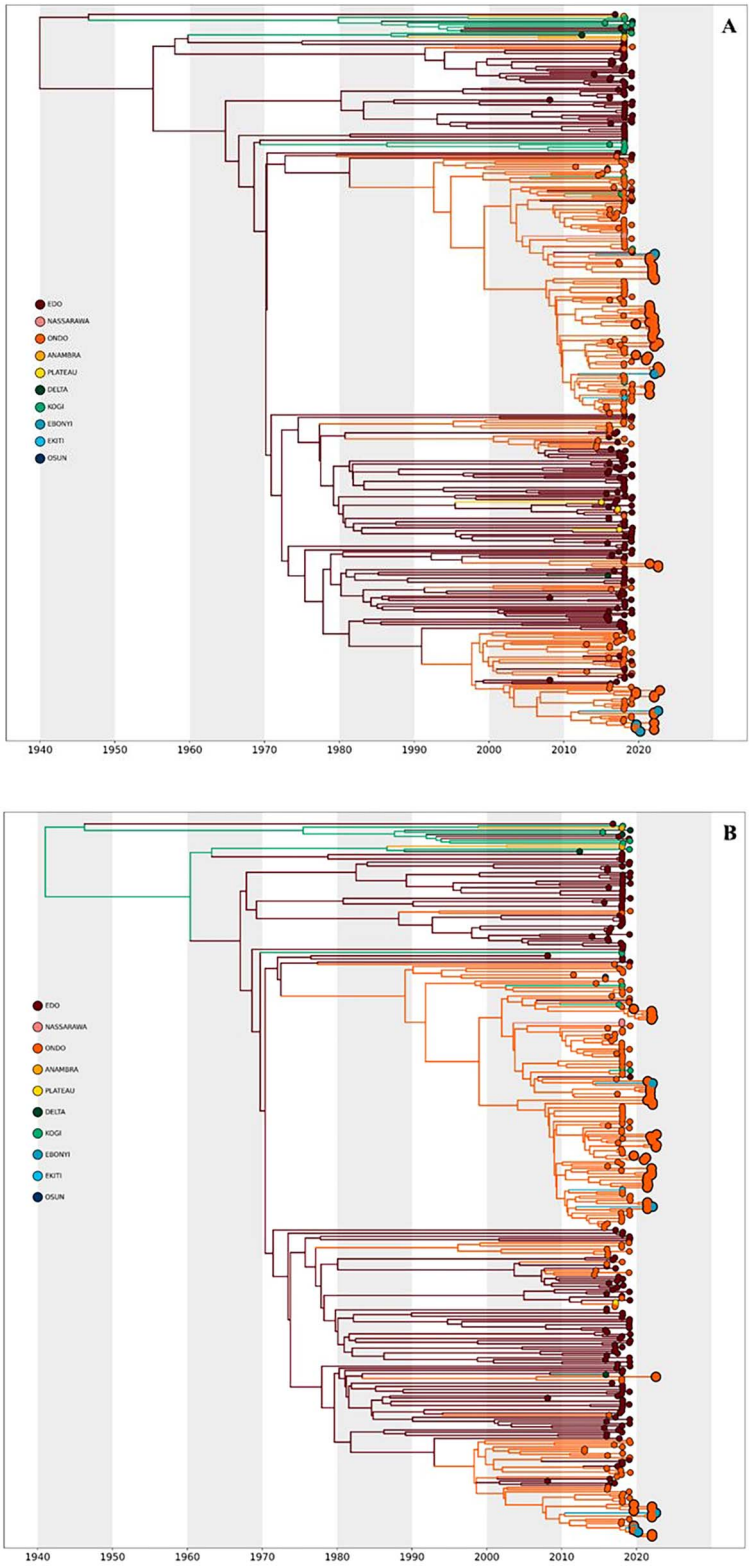

Figure 3 Time-scaled maximum clade credibility trees for LASV sublineage 2g S and L segments, annotated as per Ehichioya et al. (2019); new S (a) and L (b) segment LASV genomes generated from this study are identified by larger size bullets at the tip of the branch; sublineage 2f is included as an outgroup; each bullet at the tip of each branch indicates a Nigerian state from which the isolate was collected; the molecular clock rate for the S segment was 7.8×10^{-4} substitutions/(site $\times$ year) and 8.6×10^{-4} substitutions/(site $\times$ year) for the L segment; phylogeographic reconstruction suggests that the sublineage 2g S segment originated in Edo State, late in the year 1939 and the sublineage 2g L segment originated in Kogi State in the year 1940.

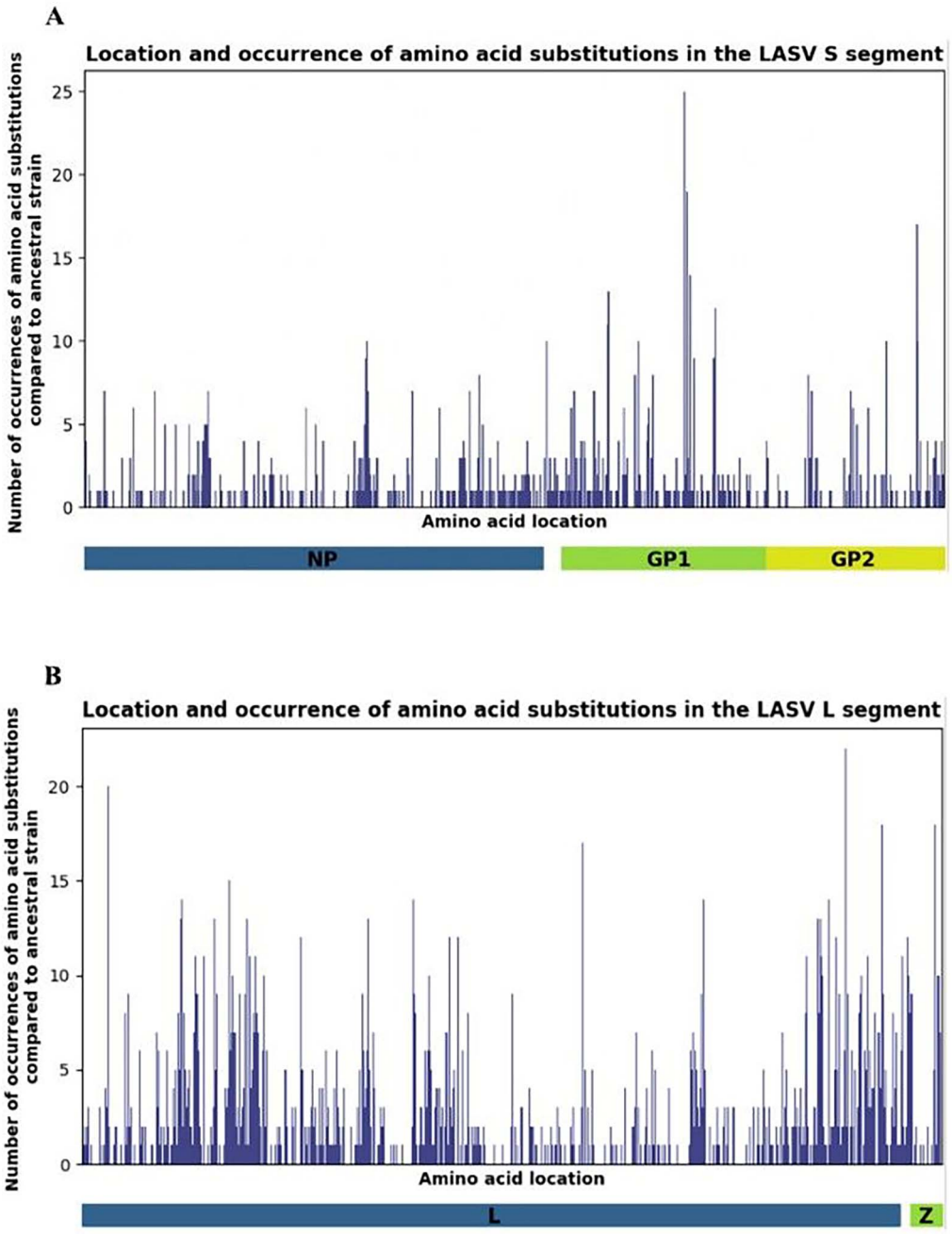

Figure 4 Location and number of amino acid substitutions in the LASV S segment (a) and L segment (b) compared to an ancestral strain.

Grammomys surdaster (4), *Mus spicilegus* (2), *Mus pahari* (11), *Rattus norvegicus* (3), *Crocidura indochinensis* (3), *R. rattus* (2), *Arvicanthis niloticus* (2), *Rhabdomys dilectus* (2), *M. natalensis* (2), *Hylomyscus alleni* (1) and *Mus musculus* (1).

Discussion

LASV PCR survey

This study demonstrates a 61.6% PCR prevalence of LASV-positive small mammals in Southern Nigeria, reproducing the substantial presence of LASV in multiple species previously reported by Happi et al. (2022). Additionally, it identifies new rodent species and shrews as potential LASV hosts, expanding the list of known reservoir or intermediate hosts (Lecompte et al. 2006, Olayemi et al. 2016, Yadouleton et al. 2019, Happi et al. 2024). Notably, in Owo, Ondo State, the study recorded a significantly higher LASV PCR positivity rate (67.1%) among sampled animals compared to Ebonyi State (53.9%). These findings correlate with previously reported human LF incidence in Ondo State (World Health Organization 2023, Nigeria Centre for Disease Control and Prevention 2025) and are evidence of the enzootic circulation of LASV. It is important to highlight that the LASV PCR positivity rate observed might have been influenced by the study sampling design. Because trapping was purposive and conducted in households

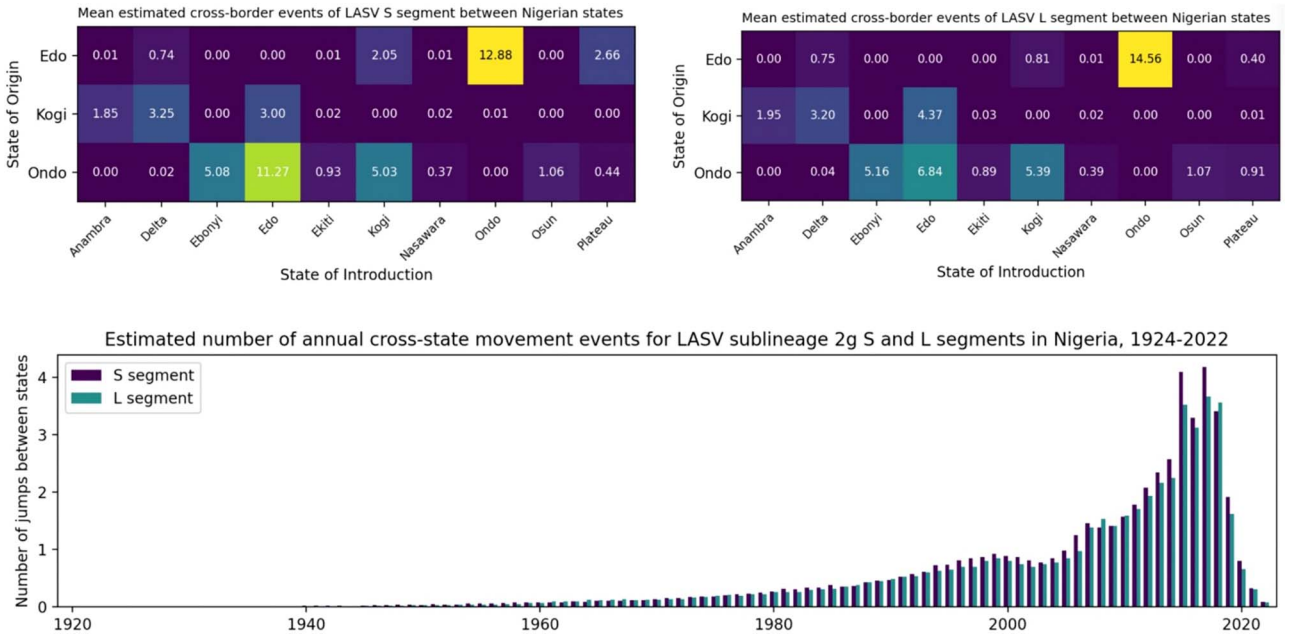

Figure 5 Mean estimated number of LASV cross-border events between Nigerian states. The mean number of instances of movement of the LASV S segment and L segment from Nigerian states of origin (y-axis) to destination states (x-axis). Estimated number of annual cross-state movement events for LASV S and L segments in Nigeria, 1924–2022. Cross-border events were identified as Markov jumps by our Bayesian phylogenetic reconstruction. All isolates represent sublineage 2g and were collected between 2008 and 2022.

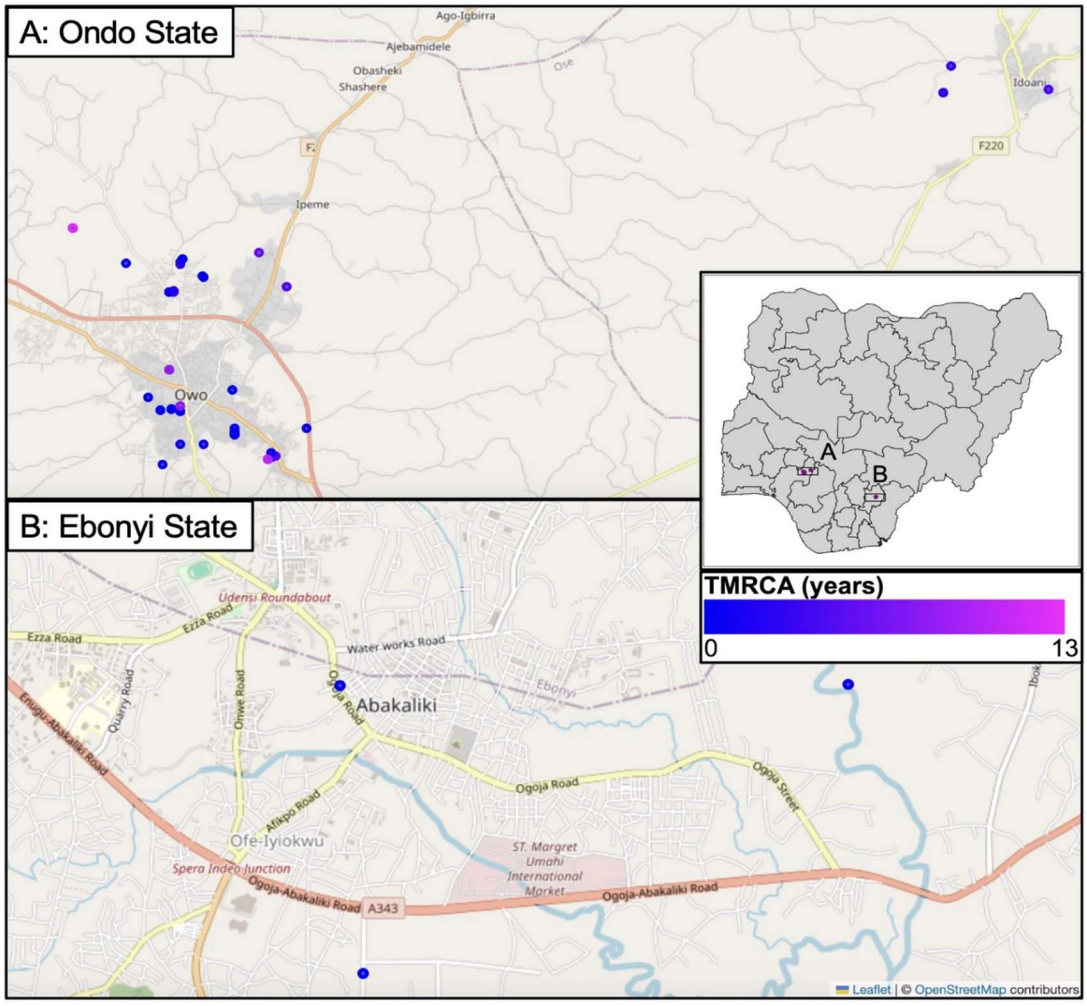

Figure 6 Map showing sites in Ondo and Ebonyi states in southern Nigeria where small mammal samples with assembled LASV genomes were generated. (The dots on the map represent each sample site with the LASV genome assembled).

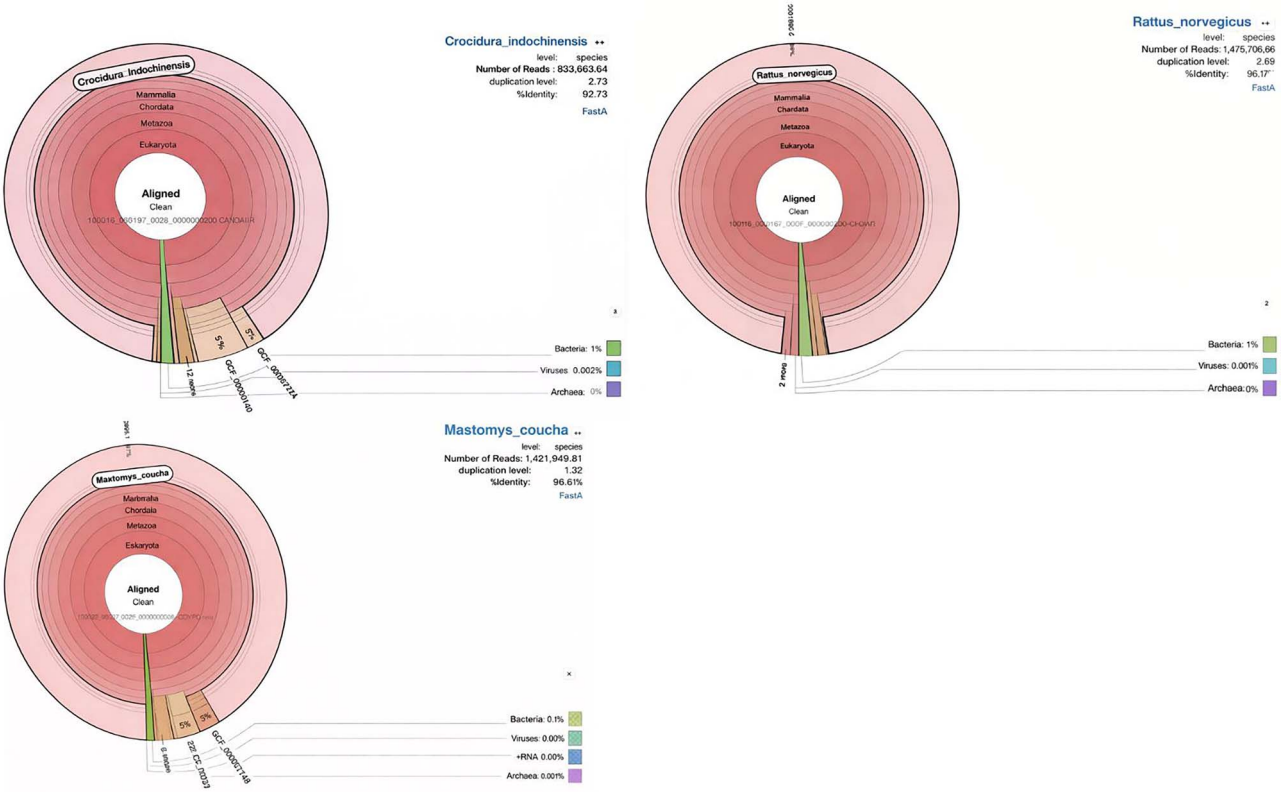

Figure 7 Three Krona charts illustrating the taxonomic identification of *Crocidura indochinensis*, *Rattus norvegicus*, and *Mastomys coucha* with 92.7%, 96.2%, and 96.6% identity, respectively.

Table 3 Mixed/crossed species of small mammals identified using NGS.

Mixed species identified	Frequency
H. alleni (67%); *M. natalensis* (33%)	2
M. natalensis (67%); *M. pahari* (33%)	1
M. natalensis (33%); *R. rattus* (17%)	3
M. musculus (50%); *M. natalensis* (50%)	1
M. natalensis (42%); *M. musculus* (11%); *M. pahari* (11%)	2
M. natalensis (20%); *M. coucha* (20%); *R. dilectus* (20%)	1
A. niloticus (20%); *M. natalensis* (20%)	2
M. natalensis (14%); *M. musculus* (14%)	1

with previously confirmed LF cases and their neighbouring dwellings, the higher detection rate in Ondo may partly reflect proximity to known human infections rather than uniform prevalence across the broader small mammal population. In addition, differences in the number of trapping sites, mammal abundance, and samples collected between states could have contributed to the observed variation. Furthermore, we used tissue samples, as opposed to blood samples in many other studies (Lecompte et al. 2006, Olayemi et al. 2016), which have reported a low LASV positivity rate by PCR in rodents. Low virus circulation in blood and high sequestration in tissues might be a major reason for this discrepancy.

Molecular evidence previously revealed that several rodent species (*M. natalensis, M. erythroleucus, H. pamfi, M. minutoides* and *M. baoulei*) may be reservoirs for LASV (Lecompte et al. 2006, Olayemi et al. 2016, Yadouleton et al. 2019, Vučak et al. 2022). Recently, we identified *Tatera* and *Crocidura* species as additional potential reservoirs (Happi et al. 2022). In this study, we further expand the list of potential LASV hosts with the identification of LASV in tissues from *M. coucha, H. alleni, M. spicilegus, M. pahari, G. surdaster, R. dilectus, A. niloticus* and *C. indochinensis*, as well as several mixed/crossed species, thereby broadening the nidality of LASV. Notably, the presence of mixed species among the sampled small mammals complicated species-level classification and represents a limitation due to the absence of close database reference matches Although it is not clear whether cross-breeding between small rodents of the same genus creates a new species.

The highest LASV prevalence (73.4%) was found in rats, reproducing a result from a recent survey conducted in Ondo (Happi et al. 2022). To date, most studies have reported *Mastomys* spp. as the most common reservoir for LASV (Monath et al. 1974, Demby et al. 2001, Fichet-Calvet et al. 2007). However, these results suggest that *Rattus* spp. and other small mammals may also have a significant role in disease ecology. This should be considered when formulating local control and prevention efforts aimed at limiting contact with LASV vectors.

Tissue samples and swabs from small mammals in Ondo had significantly lower mean LASV Ct values than those from Ebonyi. Although the link between Ct values and culturable virus presence is unclear, an inverse relationship is suggested (Singanayagam et al. 2020). One possibility is that this regional difference in Ct value is due to viral divergence in sublineage 2g, as RNA viruses mutate frequently. Higher Ct values in small mammal hosts from Ebonyi may indicate a more effective suppression of viral replication by regional hosts and an increased host tolerance to LASV infection. However, variations in

the field staff who may be adhering slightly to the sampling protocol and technique may influence viral load and detection. Experimental host, virus genomic interaction studies, and staff interchange in the two states will be required to further investigate this assumption and broaden the understanding of this significant and consistent difference in the Ct values.

LASV plasma antibody surveillance

This study found an overall anti-LASV prevalence of 45% in rodent plasma, a moderately high IgM/IgG plasma antibody-prevalence that exceeds those from previous serosurveys using ELISA (Smither et al. 2023) or immunofluorescence assay (IFA) (Fichet-Calvet et al. 2014, Bangura et al. 2021). For instance, a seropositivity of 11% was reported in *Mastomys* spp. and *Rattus* spp. in Sierra Leone using NP IgG ELISA (Smither et al. 2023). However, with IFA, seropositivity rates of 2.8% and 8% have been reported among rodents in Sierra Leone (Bangura et al. 2021) and Guinea (Fichet-Calvet et al. 2014), respectively. Despite the methodological differences, ELISA and IFA are known to produce similar sero-status classifications in rodents (Soubrier et al. 2022).

IgM antibody levels in rodents were significantly higher than IgG, suggesting active immune responses and year-round LASV infection. PCR results support continuous infection and re-infection throughout a rodent's lifetime due to persistent viral shedding. The high PCR positivity and IgM prevalence suggest many rodents were actively infected at sampling time, posing a significant risk of transmission to humans through contact with infected rodents or their excretions. The finding of simultaneous IgM and IgG in many small mammals may suggest a failure of immunoglobulin class switching, which is a known phenomenon of human LASV infection and can lead to the persistence of anti-LASV IgM for over a year after infection (Branco et al. 2011). There is the additional possibility of multiple re-infections by viral variants present in endemic areas or intermittent virions released from sequestered tissues such as the kidneys (Branco et al. 2011, Happi et al. 2022). It is worth noting that previous studies as well as the present study have demonstrated the detection and persistence of LASV in specific tissues (Happi et al. 2022, Perrett et al. 2023).

Although the dynamics of antibody development to LASV are poorly understood, this study reports a significantly higher seroprevalence of anti-GP immunoglobulin compared to anti-NP. This is likely because the glycoprotein is the only antigen displayed on the virion surface and serves as a target for neutralizing antibodies (Heinrich et al. 2020). More than half (51.2%) of the small mammals were positive for only one of NP-IgG, NP-IgM, GP-IgG, or GP-IgM, while 48.8% were positive for at least two of these targets. This heterogeneity in humoral responses to LASV infection has previously been reported in human studies, where LASV survivors may show reactivity to one or more immunological markers (Heinrich et al. 2020). This draws into question the reliability of using individual serologic markers in the diagnosis of active infection.

LASV phylogeography

In this study, our sequences from Ondo were interspersed across 10 well-supported phylogenetic clusters and two singletons in the subtree annotated as sublineage 2g (Ehichioya et al. 2019) in the S phylogeny (Fig. 3a). In the L phylogeny, our sequences cluster in five well-supported clusters and two singletons in sublineage 2g. This phylogenetic spread is expected, as LASV genetic diversity is maintained by enzootic persistence in the rodent reservoir over a wide geographic range.

Notably, this genome dataset captures for the first time the presence of LASV sublineage 2g isolated in Ebonyi State. Ebonyi is the proposed site of origin of lineage II and where sublineages 2a and 2c are known to currently co-circulate (Ehichioya et al. 2019). The presence of 2g in Ebonyi suggests either a broader distribution of sublineage 2g than previously recognized, or movement of the sublineage eastward across the Niger River from its expected range in southwestern Nigeria and against the presumed frontline of virus expansion. Given that the primary mechanism of human infection with LASV is via zoonotic transmission, an eastward expansion of the range of sublineage 2g can be presumed to be related to the movement of animal reservoirs. However, even as *M. natalensis* and other reservoir hosts are not entirely restricted by large waterways such as the Niger and Benue Rivers, the limited range of small mammals (which is typically <1 km^2) raises the concern that human or other non-rodent movement, with subsequent reverse zoonotic transmission events, may have contributed to the expansion of sublineage 2g as observed (Spiropoulou et al. 2002, van Hooft et al. 2008, Siddle et al. 2018, Klitting et al. 2022). It should be noted that discrete phylogeographic analysis requires an arbitrary collection of sampling locations that can oversimplify the reality of a studied area (Monadjem and Perrin 1998). More systematic and extensive sampling of animals in this region can better elucidate sublineage ranges and movement dynamics (van Hooft et al. 2008, Dellicour et al. 2021, Simons 2023).

Our phylogenetic clusters across both segments were closely related to sequences isolated from human cases in Owo and the wider Ondo State from 2017 to 2019. This supports the hypothesis that these human cases most likely resulted from spillover events from the common ancestors of current diversity circulating in the reservoir (Arruda et al. 2023). This also suggests a reverse zoonosis. It is important to note that human cases were often referred to Ondo State for treatment. In these cases, sampling location may not reflect the site of infection and may introduce spatial ascertainment biases. We also found that a small cluster of sequences sampled from small mammals in Owo in 2021–22 was phylogenetically incongruent across well-supported clades in the L and S segments, suggesting possible reassortment events between co-circulating lineages (Supplementary Fig. S8).

Our samples demonstrate a broader range of tMRCA for the clades defined by each isolate and the deepest node on the LASV sublineage 2g tree in Ondo State compared to Ebonyi. The range of tMRCAs for sequences from Owo is 0.1 to 12.4 years, whereas isolates from Ebonyi are all below 1 year. The presence of a greater range in tMRCA in Ondo, as well as the fact that genomes from Ebonyi are nestled within larger clusters from Ondo in the tree, suggests that the LASV in Ebonyi may represent a subpopulation of virus that was recently transferred from an older and more diverse source in Ondo.

LASV isolates originating from different hosts do not appear to form distinct clades, suggesting that the virus is consistently transmitted between species rather than being maintained in isolated populations of each species. However, the clades formed by several study isolates and deep nodes on the sublineage 2g tree appear to have tMRCA as much as 15 years prior to sampling, suggesting some degree of historical isolation between strains.

We found that our LASV sequences collected from small mammals in Ebonyi State (2019–20) clustered with sequences generated from humans sampled in Ondo State (2019–22) within sublineage 2g across

both segments, an interesting finding that may suggest inter-state movement of the virus mediated by one or more of these hosts. The mean number of cross-border movement events from Ondo to Edo States for the LASV sublineage 2g S segment was estimated to be nearly twice as high as for the L segment (Fig. 5). There were also significantly more estimated cross-border movement events of the S segment compared to the L segment from Edo to Kogi and Plateau states. This differential movement of genome segments may be due to several factors. First, there is some evidence suggesting the occurrence of reassortment between the two LASV genome segments, which could explain why the two segments have different dispersal histories (Arruda et al. 2023). Second, given that reassortment occurs, separate phylogenetic inferences were performed on BEAST for the S and L segments, with outcomes influenced by the segments' unique molecular clocks. Additionally, the underlying sequence datasets are different because some samples—both from this study and in public databases—only have sequence data for the S or L segment.

The greatest mean number of cross-border introductions was estimated to have occurred in the 4 years immediately preceding the 2018 LF epidemic, at which time 23 of Nigeria's states saw more than 3000 suspected cases and nearly 200 deaths (Andersen et al. 2015). The annual incidence of LF has remained elevated since. Though a combination of environmental, cultural-social, virological, and immune factors likely played a role in the dramatic increase, our results raise the question of whether high levels of LASV movement across states in the years before the elevated incidence set the stage for heightened exposure of humans to infected reservoirs.

The GPC gene on the S segment encodes the virion spike, which mediates viral attachment to the primary host receptor, alpha-dystroglycan DAG1 (Perrett et al. 2023). GPC is known to exhibit greater intra- and inter-host diversity than other coding regions of the LASV genome, likely due to its status as the sole antigen on the virion surface, thereby exposing it to a high degree of host selective pressure (Dalhat et al. 2022, Arruda et al. 2023). Our results from all LASV lineages confirm a larger number of amino acid substitutions, as well as a higher incidence of substitutions that confer changes in hydrophilicity, on the GPC portion of the peptide encoded by the S segment compared to those in the NP (Fig. 4; Supplementary Fig. S9). The L protein (RNApol) on the L gene of the L segment is believed to be less affected by selective pressure than the S segment because it is not exposed to hosts' defences. Although we detected a significant amount of amino acid substitution throughout the L protein of the various LASV lineages, most of these substitutions are predicted to be conservative. Whether these substitutions would influence RNApol structure or function remains to be investigated.

Our results should be interpreted within the limits of the LASV genomes generated. Most of the LASV genomes were generated from samples collected in Ondo State. Historical LASV genomes were overwhelmingly generated in Ondo and Edo. This regional focus is justified by the epidemiology of human LF, which has been concentrated in these two states since 2017 (Hastie et al. 2019). Additionally, we observed that it is very challenging to obtain LASV genomes from high Ct values from rodent samples collected in Ebonyi. To further refine our understanding of the phylogeography and diversity of LASV, sampling should be expanded more broadly to involve all regions within the endemic areas, as well as the development of targeted NGS to increase the chance of LASV genome recovery.

In conclusion, this study advances the understanding of the epidemiology and evolution of LASV within small mammal populations in Nigeria. Our serological and PCR data suggest that the virus may be present in a greater number of species than previously recognized. Furthermore, our phylogeographic identification of the emergence of sublineage 2g in Edo and Kogi states around 1940—which is now prevalent in Ondo and Ebonyi States—implies the virus's movement over several hundred kilometres and across the Niger River. This movement is potentially driven by the movement of animal hosts and infected individuals, and warrants further exploration of the role of reverse zoonosis in LASV expansion.

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Supplementary material

Supplementary material is available at *VEVOLU Journal* online.

Conflicts of interest

None declared.

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Data availability

The complete sequences of our data are available at GenBank (accession numbers from PP431155 to PP431211) and (accession number ON569369 to ON569379).

Disclaimer

Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. The research was conducted under an approved animal use protocol in compliance with DODI 3216.01, 'Use of Animals in DOD Conducted and Supported Research and Training' and other federal statutes and regulations relating to laboratory animals.

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