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Authors
Swetnam, Daniele M
Stuart, Jackson B
Young, Katherine
et al.

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Movement of St. Louis encephalitis virus in the Western United States, 2014-2018

Daniele M. Swetnam, Jackson B. Stuart, Katherine Young, Payal D. Maharaj, Ying Fang, Sandra Garcia, Christopher M. Barker, Kirk Smith, Marvin S. Godsey, Harry M. Savage, Vonnita Barton, Bethany G. Bolling, Nisha Duggal, Aaron C. Brault, Lark L. Coffey

1 Department of Pathology, Microbiology, and Immunology, School of Veterinary Medicine, University of California, Davis, California, United States of America, 2 Division of Vector-borne Diseases, Centers for Disease Control, Fort Collins, Colorado, United States of America, 3 Maricopa County Environmental Services Department, Phoenix, Arizona, United States of America, 4 Idaho Bureau of Laboratories, Boise, Idaho, United States of America, 5 Laboratory Services Section, Texas Department of State Health Services, Austin, Texas, United States of America, 6 Department of Molecular Biology, College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, United States of America

*ICoffey@ucdavis.edu

Abstract

St. Louis encephalitis virus (SLEV) is a flavivirus that circulates in an enzootic cycle between birds and mosquitoes and can also infect humans to cause febrile disease and sometimes encephalitis. Although SLEV is endemic to the United States, no activity was detected in California during the years 2004 through 2014, despite continuous surveillance in mosquitoes and sentinel chickens. In 2015, SLEV-positive mosquito pools were detected in Maricopa County, Arizona, concurrent with an outbreak of human SLEV disease. SLEV-positive mosquito pools were also detected in southeastern California and Nevada in summer 2015. From 2016 to 2018, SLEV was detected in mosquito pools throughout southern and central California, Oregon, Idaho, and Texas. To understand genetic relatedness and geographic dispersal of SLEV in the western United States since 2015, we sequenced four historical genomes (3 from California and 1 from Louisiana) and 26 contemporary SLEV genomes from mosquito pools from locations across the western US. Bayesian phylogeographic approaches were then applied to map the recent spread of SLEV. Three routes of SLEV dispersal in the western United States were identified: Arizona to southern California, Arizona to Central California, and Arizona to all locations east of the Sierra Nevada mountains. Given the topography of the Western United States, these routes may have been limited by mountain ranges that influence the movement of avian reservoirs and mosquito vectors, which probably represents the primary mechanism of SLEV dispersal. Our analysis detected repeated SLEV introductions from Arizona into southern California and limited evidence of year-to-year persistence of genomes of the same ancestry. By contrast, genetic tracing suggests that all SLEV activity since 2015 in central California is the result of a single persistent SLEV introduction. The identification of natural barriers that influence SLEV dispersal enhances our understanding of arbovirus ecology in the western United States and may also support regional public health agencies in implementing more targeted vector mitigation efforts to protect their communities more effectively.
Following the detection of West Nile virus in the United States, evidence of the historically endemic and closely related virus, St. Louis encephalitis virus (SLEV), dropped nationwide. However, in 2015, a novel genotype of SLEV, previously restricted to Argentina, was identified as the etiological agent of an outbreak of neurological disease in Arizona, United States. Since that time, the genotype has expanded throughout the Western United States, including into California, Nevada, Texas, Idaho, and Oregon. In this study, samples containing SLEV, provided by public health and mosquito abatement agencies, were sequenced and used in phylogenetic analyses to infer patterns of SLEV movement. Three independent routes of SLEV dispersal were identified: Arizona to Southern California, Arizona to Central California, and Arizona to all locations east of the Sierra Nevada mountains. The Sierra Nevada mountains and the Transverse Ranges appear to separate the three routes of SLEV movement, suggesting that geographic features may act as barriers to virus dispersal. Identification of patterns of SLEV dispersal can support regional public health agencies in improving vector mitigation efforts to protect their communities more effectively.

**Introduction**

St. Louis encephalitis virus (SLEV) is an arthropod-borne flavivirus (*Flaviviridae, Flavivirus*) maintained in an enzootic cycle involving *Culex* spp. mosquitoes and passeriform and columbiform birds. While SLEV infections are non-fatal in birds, spillover into humans [1] and horses [2] can result in significant and sometimes fatal neurological disease. The genome of SLEV is encoded by a single-stranded, positive-sense RNA genome consisting of one open reading frame (ORF) and non-coding regions at the 5' and 3' ends. The ORF is translated as a single polyprotein that is co- and post-translationally cleaved into three structural proteins and seven nonstructural (NS) proteins: Capsid (C), Envelope (E), pre-membrane (prM), NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5.

St. Louis encephalitis virus occurs throughout North and South America, as well as in the Caribbean islands [3]. Genetic variation of SLEV from different locations has been characterized using oligonucleotide fingerprinting [4], single-strand conformation polymorphism (SSCP) [5], base exclusion sequence scanning [6] and phylogenetic [7–11] methods. The most recent phylogenetic studies have classified SLEV into eight genotypes: I-VII and Palenque [7–11].

In the United States (US), sporadic focal outbreaks have been reported since SLEV was first detected in 1933 [12]. Endemic activity in the absence of outbreaks also has been reported in Florida (FL) [13], Texas (TX) [14] and California (CA) [15–17] from 1933 to 2003 (reviewed in [18]). Following the first detection of West Nile virus (WNV, also a flavivirus) in the Americas in 1999, SLEV activity was significantly reduced throughout the US [1]. In CA, SLEV was not detected after 2003, the year WNV was first detected in CA, until 2015 when SLEV-positive mosquito pools and sentinel chickens were detected in Coachella Valley in Riverside County, CA [18]. The re-emergence of SLEV detected in mosquitoes and sentinel chickens in CA was concurrent with an outbreak of human disease in Maricopa County, Arizona (AZ) [19]. Retrospective analyses of WNV-positive mosquito pools collected in Maricopa County, AZ in 2014 detected SLEV RNA in a pool, revealing that SLEV was present in that area at least one year earlier than initially detected. However, it remains unclear if SLEV was present in AZ.
prior to 2014 because SLEV was not surveyed in that state during the years leading up to the 2015 outbreak and WNV-negative pools were not saved [18].

Prior to 2014, SLEV genotypes I, II [8] and V [11,13] were reported in the US and genotypes III, IV, VI, and VII were thought to be restricted to South America [8,20]. However, recent sequencing and phylogenetic analyses have demonstrated that SLEV detected in AZ and CA during and after 2014 belong to genotype III, which was previously only reported in Argentina in 1978 and 2005 [18]. The emergence of South American SLEV genotypes in the US, now including genotype III, is consistent with previous studies that have detected periodic introductions of other SLEV genotypes from South America into North America, presumably by migrating birds [7–9].

Since 2015, SLEV has been detected each year throughout the western US, ranging from southern CA, AZ, and southern NV, to the northern Central Valley of CA and southeastern Oregon (OR) [21,22]. However, it is unclear if these detections are the result of re-emergence of previously endemic strains or whether they represent expansion of the more recently introduced genotype III SLEV. Furthermore, if the continued detection of SLEV in the western US is the result of expansion of genotype III, epidemiology and mosquito surveillance alone are not sufficient to decipher the specific routes of arbovirus spread, which may provide important insights into the ecological mechanisms influencing SLEV invasion of the western US. The western US is a geographic region that represents a heterogeneous landscape including mountains, coasts, deserts, temperate rainforests, urban cities, and farmlands, which could support endemic SLEV transmission cycles that use different hosts, vectors and mechanisms of persistence.

Given that SLEV transmission is dependent on host-vector interactions, ecological features influencing the spatial distribution of susceptible bird and mosquito species are likely to impact SLEV spread. Seroprevalence and experimental infection data show that house finches, house sparrows, common ground-doves [23] and nestling mourning doves [24] are the most important SLEV amplifying hosts in CA. House finches [25], house sparrows [26], and common ground doves [27] are resident birds that migrate short distances, while mourning doves [28] undertake long-distance migrations each year. However, banding studies that involve the capture, marking, and recapture of individual birds have demonstrated the range and overall migration distance of individual birds is highly variable even among resident birds, which have been detected more than 1000 km from their initial capture location [29].

The most important vectors for SLEV are *Culex tarsalis* [30,31] and several species in the *Cx. pipiens* complex, including *Cx. pipiens* and *Cx. quinquefasciatus* [31]. Typically, dispersal of *Cx. pipiens* [32], *Cx. quinquefasciatus* [33] and *Cx. tarsalis* is limited to distances of < 3 km; however longer-range dispersal has been reported on several occasions among female *Cx. tarsalis* mosquitoes in CA, including in the Coachella Valley of southeastern CA (5.7 km) [32] and the southern Central Valley of CA (12.6 km) [34]. Humans can also facilitate long-distance movement of mosquitoes by transport in vehicles [35].

The goal of this study was to understand SLEV movement within CA and the broader western US. We generated full ORF sequences of 4 historic SLEV isolates and 26 SLEV-positive mosquito pools collected in ecologically distinct regions of AZ, CA, NV, OR and Idaho (ID) (Fig 1) and then characterized their genetic relatedness and patterns of spread using Bayesian phylogeographic approaches. Our results show that all SLEV genomes detected in the western US since 2015 belong to genotype III, providing no evidence that any historically endemic non-genotype III SLEV continue to circulate in the western US. Within genotype III, three distinct routes of SLEV movement were detected. The routes appear to have been influenced by three mountain ranges in the western US that likely restrict the movement of SLEV mosquito vectors and avian reservoirs. The identification of these natural barriers enhances our
understanding of arbovirus ecology in the western US and may also support regional public health agencies in implementing more effective strategies for protecting their communities. For example, augmenting vector mitigation efforts in low elevation valleys, where natural barriers are more likely to be permissive to virus expansion, could prevent virus transmission into new areas. These findings also highlight the importance of collaboration between academic institutions and local public health programs in the pursuit of a more thorough understanding of infectious disease circulation.
Materials and methods

Mosquito pool sources

Mosquito pools collected in CA were procured through routine arbovirus surveillance conducted by local mosquito abatement districts and the Davis Arbovirus Research and Training laboratory at the University of California, Davis. Additional SLEV-positive mosquito pools were provided by the Texas Department of State Health Services, the Idaho Department of Health & Welfare, the Oregon Veterinary Diagnostic Laboratory, and the Southern Nevada Health District. A full list of the SLEV-positive samples, and the locations and dates of collection included in this study is shown in Table 1.

SLEV RNA extraction and genome sequencing from mosquito pools

SLEV-positive mosquito pools from CA were identified using triplex reverse transcription polymerase chain reaction (RT-PCR) [36]. Mosquito pools from other states were identified as SLEV-positive through similar molecular diagnostic approaches. Mosquito pools were homogenized in 1–2 mL of virus transport medium (VTM, which was 10% fetal bovine serum [FBS], 50 μg/mL gentamicin, 1% penicillin/streptomycin, and 5 μg/mL amphotericin B) for 2 minutes and clarified by centrifugation. Homogenized mosquito pools were filtered with a 0.45 μm syringe filter. SLEV RNA was then extracted from 140 μl of each mosquito pool filtrate using a QIAamp® Viral RNA Mini Kit (Qiagen, Hilden) in accordance with the manufacturer’s recommendations. SLEV RNA was eluted into 40 μl of nuclease free water. The extracted SLEV RNA was then amplified for sequencing using a Qiagen® OneStep Reverse transcription (RT)-Polymerase chain reaction (PCR) kit (Qiagen, Hilden). Each reaction contained 14 μl nuclease free water, 5 μl 5x RT-PCR Buffer, 1 μl dNTP Mix (10 mM of each dNTP), 1 μl RT-PCR Enzyme Mix (Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and HotStarTaq® DNA Polymerase), 0.5 μl each of ‘forward’ and ‘reverse’ primers (Table 2), and 3 μl extracted SLEV RNA. The thermal cycler conditions were as follows: a 30-minute reverse transcription step at 50˚C, a 15-minute initial PCR activation step at 95˚C, 40 cycles of a 3-step cycling phase (including a 1-minute denaturation step at 94˚C, a 1-minute annealing step at 57˚C, and a 2-minute extension step at 72˚C), and a 10-minute final extension step at 72˚C. All reactions were then held at 4˚C.

SLEV RT-PCR products were subjected to electrophoresis on 1% agarose gels and purified according to the manufacturer’s recommendations using a QIAquick® PCR Purification Kit (Qiagen, Hilden) or a QIAquick® Gel Extraction Kit (Qiagen, Hilden). Purified complementary DNA was eluted into 40 μl of Buffer EB (Qiagen, Hilden) and Sanger sequenced using the primers in Table 2 at the DNA Sequencing Core facilities at UC Davis or the Division of Vector-Borne Diseases at the Centers for Disease Control and Prevention (DVBD-CDC). The resulting sequences were aligned using a published reference sequence (GenBank accession number: KX258462) to generate a consensus sequence using Sequencher® DNA Sequence Analysis Software (Gene Codes Corporation, Ann Arbor). Prior to alignment, the sequences were trimmed using the Sequencher command “Trim Ends” in accordance with the software’s suggested trim criteria. An average of double coverage at each coding genomic position was achieved, and sequences were called by verifying that the chromatogram peaks were both clear and consistent across all strands at each nucleotide position.

Phylogenetic analyses

Consensus genomic SLEV sequences were aligned with all previously published SLEV genomes available in GenBank (Table 1) in Mega7.0.26 [37]. Only genomes that contained at
Table 1. SLEV sequences and metadata.

| Accession | Strain | Date | Location         | Source            | Genotype | Collected by |
|-----------|--------|------|------------------|-------------------|----------|--------------|
| AY632544  | Argentina 66 | 1966 | Córdoba, Argentina | Calomys musculinus | VII      | NA           |
| DQ359217  | MSI 7   | 1975 | Mississippi, US   | Unknown           | II       | NA           |
| EF158048  | BE AR 23379 | 1960 | Para, Brazil      | Sabethes belisarius | V        | NA           |
| EF158049  | 904.3   | 1955 | Kentucky, US      | Colaptes auratus  | II       | NA           |
| EF158050  | MSI 7   | 1975 | Mississippi, US   | Passer domesticus | II       | NA           |
| EF158051  | GMO 94  | 1969 | Guatemala         | Culex nigripalpus | II       | NA           |
| EF158052  | V 2380–42 | 2001 | Texas, US        | Culex quinquefasciatus | II       | NA           |
| EF158053  | BeAn 246262 | 1973 | Para, Brazil      | Didelphis marsupialis | V        | NA           |
| EF158054  | 75 D 90 | 1975 | Peru              | Unknown           | V        | NA           |
| EF158055  | THB 28  | 1962 | Florida, US       | Homo sapiens      | II       | NA           |
| EF158056  | TRVL 9464 | 1955 | Trinidad          | Psorophora ferox  | V        | NA           |
| EF158057  | 78 A 28 | 1978 | Guatemala         | Unknown           | II       | NA           |
| EF158058  | Kern 217 | 1989 | Kern County, CA, US | Culex tarsalis   | II       | NA           |
| EF158059  | 65 V 310 | 1961 | Mexico            | Unknown           | II       | NA           |
| EF158060  | GML 903797 | 1983 | Panama            | Unknown           | VI       | NA           |
| EF158061  | 69 M 1143 | 1969 | Florida           | Procyon lотор     | II       | NA           |
| EF158062  | FL 79–411 | 1979 | Florida           | Culex nigripalpus | II       | NA           |
| EF158063  | COR AN 9124 | 1966 | Córdoba, Argentina | Calomys musculinus | VII      | NA           |
| EF158064  | GML 902612 | 1973 | Panama            | Haemagogus equinus | IV       | NA           |
| EF158065  | TNM 4–711 K | 1974 | Tennessee, US     | Culex pipiens    | II       | NA           |
| EF158066  | GHA-3   | 1955 | Haiti             | Butorides virescens | II       | NA           |
| EF158067  | BE AN 247377 | 1973 | Para, Brazil      | Hylophilax poecilonota | V       | NA           |
| EF158068  | COR AN 9275 | 1967 | Córdoba, Argentina | Mus musculus      | VII      | NA           |
| EF158069  | 72 V 4749 | 1972 | Colorado, US      | Culex tarsalis   | I        | NA           |
| EF158070  | Parton 1933 | 1933 | Missouri, US      | Homo sapiens      | II       | NA           |
| EU566860  | Hubbard 1937 | 1937 | Missouri, US      | Homo sapiens (brain) | II       | NA           |
| FJ753286  | ChaAR-4005 | 2/15/05 | Córdoba, Argentina | Culex quinquefasciatus | III      | NA           |
| FJ753287  | 79V-2533 | 1978 | Santa Fe, Argentina | Culex spp.      | III       | NA           |
| JF460777  | IMP115 | 2003 | CA, US            | Culex tarsalis   | V        | NA           |
| JQ957868  | Palenque- C475  | 2008 | Mexico            | Culex nigripalpus | PAL      | NA           |
| JQ957869  | Palenque- A770  | 2008 | Mexico            | Culex nigripalpus | PAL      | NA           |
| KF589299  | FLU3632 | 3/27/06 | Peru        | Homo sapiens (oropharyngeal swab) | V       | NA           |
| KM267635  | BeH355964 | 1978 | Para, Brazil      | Homo sapiens      | V        | NA           |
| KT823415  | RT121B  | 7/7/15 | Maricopa County, AZ | Culex quinquefasciatus | III      | NA           |
| KX258460  | AZ43    | 7/14/15 | Maricopa County, AZ | Culex tarsalis   | III       | NA           |
| KX258461  | COAV2281 | 7/28/15 | Coachella Valley, CA, US | Culex tarsalis     | III       | NA           |
| KX258462  | AZ39    | 7/14/15 | Maricopa County, AZ | Culex quinquefasciatus | III      | NA           |

(Continued)
Table 1. (Continued)

| Accession | Strain | Date     | Location            | Source                     | Genotype | Collected by                          |
|-----------|--------|----------|---------------------|----------------------------|----------|---------------------------------------|
| KX965720  | AZ14   | 2014     | AZ, US              | Culex spp.                 | III      | NA                                    |
| KY825742  | KERN2  | 2016     | Kern County, CA, US | Culex pipiens              | III      | NA                                    |
| KY825743  | KERN1  | 9/9/16   | Kern County, CA, US | Homo sapiens               | III      | NA                                    |
| MN233306  | RT280† | 6/15/17  | Maricopa, AZ, US    | Culex tarsalis             | III      | Maricopa County Environmental Services Department |
| MN233307  | BUCO327⁺ | 8/28/17  | Butte County, CA, US | Culex tarsalis             | III      | Butte County Mosquito and Vector Control District |
| MN233308  | COAV3064⁺ | 7/26/17  | Coachella Valley, CA, US | Culex tarsalis             | III      | Coachella Valley Mosquito and Vector Control District |
| MN233309  | DLNO22⁺ | 9/15/17  | Delano County, CA, US | Culex quinquefasciatus     | III      | Delano Mosquito Abatement District     |
| MN233310  | FRWS650⁺ | 10/12/17 | Fresno Westside, CA, US | Culex tarsalis             | III      | Fresno Westside Mosquito Abatement District |
| MN233311  | ID17⁺  | 9/12/17  | Gem County, ID, US   | Culex tarsalis             | III      | Idaho Bureau of Laboratories          |
| MN233312  | IMPR165⁺ | 7/20/18  | Imperial County, CA, US | Culex quinquefasciatus     | III      | Imperial County Vector Control       |
| MN233313  | IMPR570⁺ | 9/11/17  | Imperial County, CA, US | Culex tarsalis             | III      | Imperial County Vector Control       |
| MN233314  | KERN245⁺ | 7/5/18   | Kern County, CA, US  | Culex quinquefasciatus     | III      | Kern Mosquito and Vector Control District |
| MN233315  | KERN345⁺ | 7/15/16  | Kern County, CA, US  | Culex quinquefasciatus     | III      | Kern Mosquito and Vector Control District |
| MN233316  | KERN351⁺ | 6/21/17  | Kern County, CA, US  | Culex quinquefasciatus     | III      | Kern Mosquito and Vector Control District |
| MN233317  | MADR393⁺ | 9/29/17  | Madera County, CA, US | Culex quinquefasciatus     | III      | Madera County Mosquito and Vector Control District |
| MN233318  | MERC342⁺ | 9/14/17  | Merced County, CA, US | Culex tarsalis             | III      | Merced County Mosquito Abatement District |
| MN233319  | NV16⁺ | 5/15/16  | Clark County, NV, US | Culex tarsalis             | III      | Southern Nevada Health District, Environmental Health Public Accommodations & Mosquito Disease Surveillance |
| MN233320  | OR17⁺ | 2017     | Malheur County, OR, US | Culex spp.              | III      | Oregon Veterinary Diagnostic Laboratory |
| MN233321  | SUYA288⁺ | 7/31/17  | Sutter/Yuba County, CA, US | Culex tarsalis             | III      | Sutter-Yuba Mosquito and Vector Control District |
| MN233322  | TLRE179⁺ | 8/16/17  | Tulare, CA, US       | Culex quinquefasciatus     | III      | Tulare Mosquito Abatement District     |
| MN233323  | TRLK660⁺ | 8/3/17   | Turlock, CA, US      | Culex pipiens              | III      | Turlock Mosquito Abatement District    |
| MN233324  | AR15-6004⁺ | 7/21/2015 | El Paso, TX, US    | Culex quinquefasciatus     | III      | Texas Department of State Health Services, Arbovirus-Entomology Laboratory |
| MN233325  | WEST13⁺ | 7/20/16  | Kern County, CA, US  | Culex tarsalis             | III      | West Side Mosquito and Vector Control District |
| MN233326  | COAV2623⁺ | 8/25/15  | Coachella Valley, CA, US | Culex tarsalis             | III      | Coachella Valley Mosquito and Vector Control District |
| MN233327  | COAV2361⁺ | 8/4/15   | Coachella Valley, CA, US | Culex tarsalis             | III      | Coachella Valley Mosquito and Vector Control District |
| MN233328  | COAV2616⁺ | 8/25/15  | Coachella Valley, CA, US | Culex tarsalis             | III      | Coachella Valley Mosquito and Vector Control District |
| MN233329  | RT496⁺ | 7/10/15  | Maricopa, AZ, US     | Culex quinquefasciatus     | III      | Maricopa County Environmental Services Department |
| MN233330  | RT246⁺ | 7/21/15  | Maricopa, AZ, US     | Culex quinquefasciatus     | III      | Maricopa County Environmental Services Department |

(Continued)
least 99% of the SLEV ORF were included in the alignment. A nucleotide substitution model was identified by comparing 88 models using Akaike and Bayesian information criterion in jModelTest2 [38] on a CIPRES Science Gateway [39]. The evolutionary history was inferred by using a Maximum Likelihood method in Mega7.0.26 [37]. The tree with the highest log-likelihood of 500 bootstraps is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. The tree was visualized in FigTree v1.4.3 and rooted using the midpoint root function.

**Phylogeographic analyses**

Bayesian phylogeographic approaches were used to investigate the spatial expansion of SLEV since 2015. Since Bayesian phylogenetic methods incorporate time into their reconstructions, the alignment was first evaluated to determine the strength of the temporal signal. The temporal signal was evaluated by comparing the collection date of each mosquito pool with the phylogenetic distance (root-to-tip distance) in the Maximum Likelihood tree in TempEST [40].

### Table 1. (Continued)

| Accession | Strain | Date       | Location                      | Source                  | Genotype | Collected by                      |
|-----------|--------|------------|-------------------------------|-------------------------|----------|-----------------------------------|
| MN233331  | LA-01-4278* | 8/30/2001  | Ouachita Parish, LA, US       | *Culex quinquefasciatus* | II       | Center for Disease Control       |
| MN233332  | BFS1750*  | 1993       | Kern County, CA, US           | *Culex tarsalis*        | I        | Kern Mosquito and Vector Control District |
| MN233333  | COAV750*  | 1998       | Coachella Valley, CA, US      | *Culex tarsalis*        | I        | Coachella Valley Mosquito and Vector Control District |
| MN233334  | KERN217*  | 1989       | Kern County, CA, US           | *Culex tarsalis*        | II       | Kern Mosquito and Vector Control District |
| MN233335  | TLRE15*   | 6/20/18    | Tulare, CA, US                | *Culex quinquefasciatus* | III      | Maricopa County Environmental Services Department |

The accession number and strain name of each sequence used in this study are summarized with the associated genotype and metadata including location and date. Where possible, the full date including month and day was provided (mm/dd/yyyy). Novel genomes generated in this study are indicated with an asterisk (*).

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### Table 2. Primers used to amplify and sequence St. Louis encephalitis virus.

| Forward primer name | Forward primer sequence | Reverse primer name | Reverse primer sequence |
|---------------------|-------------------------|---------------------|-------------------------|
| F1                  | GAGCGGAGAGGAAAAAAGATTT  | 800R                | AAAGAGATGTGTGAGCCGT     |
| 636p                | GCATGGGACATTCAAGGCCG    | 1963n               | GACCGTGCAATCTCCAAA      |
| 1874p               | TACACTGGAAGCAGGAGACC    | 3145n               | TTAGGGCCGCCTAGTTA       |
| 3068p               | CCAGAAGACCCACACCTATG    | 3784n               | AGCTGCTCAAATAAACATCA    |
| 3689p               | GCTGTCTCAAAGTGCAACC     | 4999n               | ACCCTGTCAAATCTAGTACC    |
| 4893p               | GAGCCGCTGACTGTATTCCC    | 6182n               | CGTTGGAGGCCACCTTTGAAAG  |
| 5958p               | ATGAGGAGCCAGCAGATTGG    | 7216n               | GCATTATGATCCAGGATGG     |
| 7125p               | TGCTGGGTTGTTGGAATCAA    | 8386n               | ATGTAATTTGGAAGTGGAACG   |
| 8314p               | CATGGGAAAGGATGGCCAAACAG | 9009n              | GGAATTTGGGAGGCCTAAAGG   |
| 9000F               | CCAAAATCTGCGGAATAGGTT   | R6                  | ATTTCAACGAGGACAGATG     |
| F7                  | GTTGTAGTGCGTAGGAGAACAA  | R14                 | TAAACGCGTGTCTCTGAAACC   |

Each row represents a primer pair.

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The relationship between collection date and root-to-tip distance of each genome was compared using linear regression and a Pearson correlation test in R version 3.5.3 [41].

Genomes belonging to SLEV genotype III were used to further investigate the geospatial diffusion patterns of SLEV in the western US using a Bayesian phylogeographic platform implemented through BEAST v1.10.4 [42]. Two partial SLEV genomes from AZ, AZ14 (GenBank accession number: KX965720) and RT246 (GenBank accession number: MN233330), for which approximately 85% and 90% of the genome, respectively, were available, were also included to maximize use of all available SLEV genomic information.

Standard path-sampling and stepping-stone approaches were used to determine the optimal combination of clock model (fixed molecular clock), tree prior (Bayesian skyline) and continuous trait diffusion model (Cauchy). Each Markov Chain Monte Carlo (MCMC) process was sampled for 50 million steps, and every 5,000th step was recorded. Log files were inspected visually in Tracer to confirm that each prior underwent adequate mixing and the MCMC chain achieved topological convergence. Three independent MCMC chains were combined in LogCombiner and 10% burn-in was removed. Maximum clade credibility trees were annotated in TreeAnnotator. Both LogCombiner and TreeAnnotator are available through the BEAST v1.10.4 package [42]. Phylogenies were visualized in FigTree and geospatial reconstruction was performed in SpreaD3 [43].

Map preparation and statistical testing
Mapping and statistical testing were performed in R version 3.5.3 [41]. Maps were generated using the following packages: dismo, ggplot, rasrer, rnaturalearth, rnaturalearthdata and sf. Elevation and temperature data were data provided by WorldClim version 2 (resolution 2.5 minutes) and represent means for years 1970–2000 [44].

Results
Genome sequencing of SLEV from the Western United States, 2015–2018
The full ORF sequences were determined for 26 SLEV-positive mosquito pools from 3 species collected from 2015–2018 in CA (n = 19), TX (n = 1), NV (n = 1), AZ (n = 3), OR (n = 1) and, ID (n = 1) (Table 1). Additional ORF sequences from four historical SLEV strains were also determined and included: BFS1750 (Kern County, CA 1953), COAV750 (Coachella Valley, CA 1988), KERN217 (Kern County, CA 1989), and LA-01-4278 (Monroe, Louisiana 2001). The consensus sequences generated in this study were aligned with 40 previously published SLEV genomes and were deposited in GenBank (GenBank accession numbers: MN233306-MN233335).

Phylogenetic analyses
The evolutionary history of SLEV genomes was inferred with Maximum Likelihood analyses (Fig 2A). Eight SLEV genotypes were identified and the clustering pattern of all published sequences was consistent with previous reports (Fig 2) [7–11]. All SLEV genomes collected after 2014 clustered together in genotype III, along with two Argentinian sequences (strain name and GenBank accession numbers: CbaAr-4005: FJ753286 and 79V-2533: FJ753287) and six previously published SLEV genomes collected in the Western US (strain name and GenBank accession numbers: AZ14: KX965720, AZ39: KX258462, AZ43: KX258460, COAV2281: KX258461, KERN1: KY825743, KERN2: KY825742). This clustering pattern strongly suggests there was a single introduction of genotype III SLEV from South America into North America, and that all genomes from the western US since 2014 are descendants of a single genotype III.
ancestor. The historic SLEV genomes clustered in genotypes I (BFS1750 and COAV750) and II (LA01 and KERN217) (Fig 2A). The most appropriate nucleotide substitution model was identified as a general time reversible model with a gamma shape parameter and proportion of invariable sites (GTR + G + I) by Akaike and Bayesian Information Criterion by jModelTest2 (Table 3). The evolutionary history of SLEV genomes was inferred with Maximum Likelihood analyses (Fig 2A).

Given that Maximum Likelihood phylogenies are naive to time, the root-to-tip distance of each sequence was compared with the collection date of each genome to measure the strength of the temporal signal within the phylogeny and to determine if time-aware methods, as applied in Bayesian phylogenetic approaches, are appropriate. Unfortunately, the temporal signal was insufficient to allow further analysis using Bayesian methods (Correlation coefficient = -0.36, p-value = 0.0031). However, the temporal strength was sufficiently strong (Correlation coefficient = 0.99, p-value < 2.2e-16) when the phylogeny only contained sequences belonging to genotype III (Fig 2B).

Phylogeographic analyses

The evolutionary histories of the genotype III SLEV sequences were further investigated using a Bayesian approach which incorporates sampling time into reconstructions. The SLEV genomes after 2014 clustered into four groups which we are identifying as IIIa-d (Fig 3). The clustering pattern of the Bayesian phylogeny supported the results obtained using Maximum Likelihood methods. Greater resolution was achieved using the Bayesian model, which is not surprising as the method allows for the inclusion of priors and selects heavily against polytomies. The most recent common ancestor occurred in approximately March of 2013 (95% highest posterior density (HPD) 2012.7 and 2013.8). Clusters IIIa and IIIc originated in AZ and spread into southern CA while remaining south of the Transverse Ranges that form the southern boundary of the Central Valley. Cluster IIIa (posterior = 1) comprises genomes from the southwestern US, including AZ and southern CA from 2015 until 2018, three genomes from AZ in 2015, one from AZ in 2017, and three from southern CA in 2015–2018. Cluster IIIc (posterior = 1) appeared to be geographically restricted, only containing genomes from mosquito pools from Coachella Valley, Riverside County, in southern CA during 2015. Cluster IIIb (posterior = 0.2) contained three genomes from AZ from 2014–2015, as well as all genomes from east of the Sierra Nevada mountains (NV 2016, TX 2015, ID 2017, and OR 2017). However, given the poor support of cluster IIIb, it is unclear how the genomes in this cluster are related to each other. Finally, cluster IIIId (posterior = 1) contained 14 genomes from 2016 and 2018 in the Central Valley of CA, which is surrounded by mountain ranges.

Sixty-three amino acid differences were identified among all of the North American SLEV isolates compared to the 2005 genotype III strain from Argentina, CbaAR-4005 (Table 4). All North American genotype III SLEV genomes differed from CbaAR-4005 by three amino acid substitutions: prM V-140-A, NS2A G-79-S, and NS5 I-107-V, and all but one genome (RT246)
### Table 3. Nucleotide Substitution Model Selection.

| Model          | Negative log-likelihood | BIC          | AIC          |
|----------------|-------------------------|--------------|--------------|
| F81            | 57977.882               | 117258.494   | 116237.764   |
| F81+G          | 55570.1789              | 112452.327   | 111424.358   |
| F81+I          | 55661.7749              | 112635.519   | 111607.55    |
| F81+I+G        | 55565.9171              | 112453.043   | 111417.834   |
| GTR            | 52851.1233              | 107051.173   | 105994.247   |
| GTR+G          | 50384.2976              | 102126.752   | 101062.595   |
| GTR+I          | 50439.7134              | 102237.592   | 101173.427   |
| GTR+I+G        | 50359.8932              | 102087.191   | 101015.786   |
| HKY            | 53448.9191              | 108209.807   | 107181.838   |
| HKY+G          | 50791.6354              | 102904.479   | 101869.271   |
| HKY+I          | 50905.2909              | 103131.79    | 102096.582   |
| HKY+I+G        | 50781.5802              | 102893.608   | 101851.16    |
| JC             | 58008.1073              | 117291.227   | 116292.215   |
| JC+G           | 55585.8946              | 112456.041   | 111449.789   |
| JC+I           | 55679.6947              | 112643.641   | 111637.389   |
| JC+I+G         | 55581.5697              | 112456.63    | 111443.139   |
| K80            | 53483.8306              | 108251.913   | 107245.661   |
| K80+G          | 50812.464               | 102918.419   | 101904.928   |
| K80+I          | 50930.0426              | 103153.576   | 102140.085   |
| K80+I+G        | 50803.7718              | 102910.274   | 101889.544   |
| SYM            | 52980.1957              | 107281.6     | 106246.391   |
| SYM+G          | 50504.7013              | 102339.85    | 101297.403   |
| SYM+I          | 50566.1488              | 102462.745   | 101420.298   |
| SYM+I+G        | 50485.084               | 102309.855   | 101260.168   |
| TIM1           | 52909.0319              | 107148.511   | 106106.064   |
| TIM1+G         | 50449.015               | 102237.717   | 101188.03    |
| TIM1+I         | 50507.2858              | 102354.258   | 101304.572   |
| TIM1+I+G       | 50427.7324              | 102204.391   | 101147.465   |
| TIM1ef         | 53043.7432              | 107390.216   | 106369.486   |
| TIM1ef+G       | 50572.9905              | 102457.95    | 101429.981   |
| TIM1ef+I       | 50635.1094              | 102582.188   | 101554.219   |
| TIM1ef+I+G     | 50554.9754              | 102431.159   | 101395.951   |
| TIM2           | 52864.7698              | 107059.987   | 106017.54    |
| TIM2+G         | 50414.228               | 102168.143   | 101118.456   |
| TIM2+I         | 50474.6248              | 102288.936   | 101239.25    |
| TIM2+I+G       | 50392.3532              | 102133.632   | 101076.706   |
| TIM2ef         | 52995.1456              | 107293.021   | 106272.291   |
| TIM2ef+G       | 50530.5464              | 102373.062   | 101345.093   |
| TIM2ef+I       | 50595.7329              | 102503.435   | 101475.466   |
| TIM2ef+I+G     | 50512.0088              | 102345.226   | 101310.018   |
| TIM3           | 52914.6032              | 107159.654   | 106117.206   |
| TIM3+G         | 50435.4302              | 102210.547   | 101160.86    |
| TIM3+I         | 50486.883               | 102313.453   | 101263.766   |
| TIM3+I+G       | 50410.8649              | 102170.656   | 101113.73    |
| TIM3ef         | 53048.1007              | 107398.931   | 106378.201   |
| TIM3ef+G       | 50564.0983              | 102440.166   | 101412.197   |
| TIM3ef+I       | 50620.5372              | 102553.043   | 101525.074   |

(Continued)
Table 3. (Continued)

| Model       | Negative log-likelihood | BIC     | AIC     |
|-------------|-------------------------|---------|---------|
| TIM3ef+I+G  | 50444.1956              | 102409.6| 101374.391|
| TPM1        | 53476.7759              | 108247.043| 107233.552|
| TPM1+G      | 50807.4364              | 102917.603| 101896.873|
| TPM1+I      | 50925.9805              | 103154.691| 102133.961|
| TPM1+I+G    | 50798.7447              | 102909.459| 101881.489|
| TPM1uf      | 53441.925               | 108205.058| 107169.85|
| TPM1uf+G    | 50787.1936              | 102904.835| 101862.387|
| TPM1uf+I    | 50901.7959              | 103134.039| 102091.592|
| TPM1uf+I+G  | 50777.1414              | 102893.97| 101844.283|
| TPM2        | 53428.3098              | 108150.11| 107136.62|
| TPM2+G      | 50766.4113              | 102835.552| 101814.823|
| TPM2+I      | 50890.9469              | 103084.624| 102063.894|
| TPM2+I+G    | 50757.7144              | 102827.398| 101799.429|
| TPM2uf      | 53397.824               | 108116.856| 107081.648|
| TPM2uf+G    | 50756.8585              | 102844.165| 101801.717|
| TPM2uf+I    | 50875.119               | 103080.686| 102038.238|
| TPM2uf+I+G  | 50747.0984              | 102833.884| 101784.197|
| TPM3        | 53481.5337              | 108256.558| 107243.067|
| TPM3+G      | 50797.4994              | 102897.729| 101876.999|
| TPM3+I      | 50912.7576              | 103128.245| 102107.515|
| TPM3+I+G    | 50788.2729              | 102888.515| 101860.546|
| TPM3uf      | 53448.2772              | 108217.763| 107182.554|
| TPM3uf+G    | 50775.4394              | 102881.326| 101838.879|
| TPM3uf+I    | 50886.6648              | 103103.777| 102061.33|
| TPM3uf+I+G  | 50764.6676              | 102869.022| 101819.335|
| TrN         | 52915.8735              | 107152.955| 106117.747|
| TrN+G       | 50453.7711              | 102237.99| 101195.542|
| TrN+I       | 50511.025               | 102352.498| 101310.05|
| TrN+I+G     | 50432.1668              | 102204.02| 101154.334|
| TrNef       | 53050.7523              | 107394.995| 106381.505|
| TrNef+G     | 50578.1623              | 102459.054| 101438.325|
| TrNef+I     | 50639.247               | 102581.224| 101560.494|
| TrNef+I+G   | 50559.8385              | 102431.646| 101403.677|
| TVM         | 53384.7168              | 108109.12| 107059.434|
| TVM+G       | 50729.3802              | 102807.686| 101750.76|
| TVM+I       | 50847.0239              | 103042.974| 101986.048|
| TVM+I+G     | 50718.848               | 102795.861| 101731.696|
| TVMef       | 53414.8055              | 108141.58| 107113.611|
| TVMef+G     | 50739.9071              | 102801.022| 101765.814|
| TVMef+I     | 50862.8135              | 103046.835| 102011.627|
| TVMef+I+G   | 50730.6555              | 102791.759| 101749.311|

Summary of all models compared using Bayesian and Akaike Information Criterion.

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contained the amino acid substitution NS4A L-18-S. Four genomes within cluster IIIa (AZ17, COAV3064, IMPR165 and IMPR570) shared the amino acid change NS4A G-96-E. Two genomes within cluster IIIb: ID17 and OR17, shared amino acid substitution NS1 H-216-Y and NS4A I-41-V. All genomes in cluster IIIc shared amino acid substitution NS5 I-496-V. All
genomes within cluster IIId contained an amino acid substitution NS4A P-106-S and seven of the genomes within cluster IIId contained a second amino acid substitution, E Q-175-R. While it is possible that some or all of these amino acid substitutions arose from stochastic variation, additional studies are needed to determine if they confer a change in infectivity or transmissibility of SLEV in the western US.

To define the geographic spread of SLEV in the western United States since 2014, a phylogeographic reconstruction was performed (Fig 4). All North American genotype III SLEV strains originated in AZ and three independent routes of SLEV expansion were identified. One route originated in AZ and expanded westward into Southern CA, and two routes projected northward on either side of the Sierra Nevada mountains.

**Discussion**

SLEV is a re-emerging arthropod-borne virus that has caused significant outbreaks throughout the western US in recent years. To understand the recent re-emergence and spread of SLEV in
Table 4. Summary of amino acid substitutions between SLEV strain CbaAr-4005 (GenBank accession number FJ753286) and the SLEV genomes sequenced in the present study.

| Protein | Position | Strain | Polyprotein Location |
|---------|----------|--------|----------------------|
| A       | 71       | 71     | C                    |
| AZ43    | 71       | 71     | C                    |
| AZ39    | 71       | 71     | C                    |
| RT246   | 71       | 71     | C                    |
| AZ17    | 71       | 71     | C                    |
| COAV306 | 71       | 71     | C                    |
| IMPR165 | 71       | 71     | C                    |
| IMPR570 | 71       | 71     | C                    |
| COAV2281| 71       | 71     | C                    |
| COAV2616| 71       | 71     | C                    |
| COAV2361| 71       | 71     | C                    |
| COAV2623| 71       | 71     | C                    |
| RT496   | 71       | 71     | C                    |
| AZ14    | 71       | 71     | C                    |
| NV16    | 71       | 71     | C                    |
| TX15    | 71       | 71     | C                    |
| RT121B  | 71       | 71     | C                    |
| ID17    | 71       | 71     | C                    |
| OR17    | 71       | 71     | C                    |
| KERN351 | 71       | 71     | C                    |
| BUCO327 | 71       | 71     | C                    |
| DLNO229 | 71       | 71     | C                    |
| FRWS650 | 71       | 71     | C                    |
| MADR393 | 71       | 71     | C                    |
| MERC342 | 71       | 71     | C                    |
| SUYA288 | 71       | 71     | C                    |
| TRLK660 | 71       | 71     | C                    |
| WEST13  | 71       | 71     | C                    |
| CbaAr-4005 | | | |

A dot indicates no amino acid difference between strains. Asterisks indicate sequencing gaps where the amino acid identity at the position is unknown. Protein and position indicate the amino acid position based on the Beh355964 genome (GenBank accession number KM267635), while the polyprotein number indicates the amino acid position relative to the CbaAr-4005 polyprotein.

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the western US, 30 SLEV genomes were sequenced and characterized using phylogeographic approaches. All SLEV strains detected in the US prior to 2014 clustered in genotypes I, II or V [8,11,13], but all SLEV available for this study during and after 2014 clustered monophyletically in genotype III with the 1978 and 2005 Argentinian genomes. The most recent common ancestor of genotype III SLEV in the western US was estimated to have arisen around March of 2013, suggesting that all genotype III SLEV in the US is the result of a single introduction that occurred between August 2012 and October 2013. All three of the genotypes historically endemic to the US (genotypes I, II and V) were also identified in CA during 1955 and 1983, 1989, and 2003, respectively (Fig 2A). Due to the limited number of sequences available, it is unclear if these genomes circulated concurrently or sequentially in time.

Following the introduction of genotype III, SLEV has accrued multiple amino acid changes. Sixty-three amino acid substitutions were identified in genotype III SLEV genomes from the western US. Four amino acid substitutions were conserved among almost all genotype III sequences from the western US and five were conserved along internal phylogenetic branches. Additional studies are needed to determine if these substitutions arose stochastically or in response to local selection pressures.

The SLEV sequences from the western US studied here form four geographically distinct clusters, IIIa-d (Fig 3), in genotype III, and appear to have traveled along three independent routes (Fig 4). Support for three of the clades, IIIa, IIIc, and IIId, was very strong (posterior ~1); however, support for cluster IIId was much weaker (posterior <0.2) and should be interpreted with caution. Further studies with additional genomes may help to clarify the relationships within the clusters.

The pattern of SLEV expansion suggests that mountain ranges; specifically, the Sierra Nevada, Cascade, and the Transverse Ranges (Fig 1B) have acted as natural barriers to the geographic expansion of SLEV in the Western US. This observation is consistent with limited evidence that suggests that WNV, which cycles in a bird-mosquito-bird pattern as well, has also been geographically restricted in a similar way [45,46]. While both WNV and SLEV are maintained in passerine birds throughout the US, WNV infects a wider range of bird species and...
causes more significant disease. Despite the broader host range, phylogenetic studies have demonstrated that WNV sequences from CA cluster together, suggesting a limited number of introductions into or out of the state (45, 46). Similar studies have also found that the westward expansion of WNV appears to have stalled upon reaching the Rocky Mountains (45). Together, these studies suggest that mountain ranges likely inhibit arbovirus expansion in general by influencing movement of vector mosquito and reservoir bird species. Elevation gradients (Fig 1B) are associated with rapid changes in temperature (Fig 1C), vegetation, land use and precipitation, all of which significantly impact and often restrict the distribution of mosquito and bird populations.

In the western US, the Tehachapi Mountains in the Transverse Range [47,48] as well as the Cascade [47–49] and Sierra Nevada ranges [47–49] limit gene flow among populations of Cx. tarsalis. In southeastern CA, Cx. tarsalis abundance is inversely related to elevation [50]. Genetic structure has also been reported within the Cx. pipiens complex in the western US. Cx. quinquefasciatus are restricted south of the Tehachapi Mountains, Cx. pipiens are found in northern CA (north of 39˚N) (Fig 1A), and a hybrid zone of the two species is found in the Central Valley of CA [51–55]. Given that Cx. pipiens quinquefasciatus hybrids are more efficient transmitters of the closely related WNV than either Cx. pipiens or Cx. quinquefasciatus [56], it is possible that variation in vector competence among mosquitoes in the Cx. pipiens complex has also influenced the expansion of SLEV, especially because no SLEV-positive mosquito pools have been detected in CA north of the 40˚N (Fig 1A) [22] where Cx. pipiens are most prevalent.

The relationship between mountain ranges and SLEV dispersal is further complicated by involvement of the avian hosts, passeriform and columbiform birds. While birds are more mobile than mosquitoes, there is evidence that the Sierra Nevada, Cascade, and Transverse Mountain Ranges are also barriers for gene flow within some passerine bird species [57–60], where mountains restrict bird dispersal within breeding grounds. Mountain ranges influence the migration of passerine birds in the western US as elevation gradients drive seasonal fluctuations in ecological productivity [61]. Considering the well-documented effect of elevation gradients on gene flow and migration of Passeriform birds, as well as the geographic variation in the abundance of SLEV susceptible mosquitoes, reduced SLEV dispersal across mountain ranges, deserts, or other environments unsuitable for reservoir hosts or vectors can be expected [51–55,62].

In addition, the Mojave and Colorado deserts in the southwestern US may have also contributed to the pattern of SLEV expansion. Mosquito populations rely on the maintenance of aqueous larval habitats originating from natural (winter rainfall, expansion of salt marshes along the Salton Sea, CA) and anthropogenic (residential and agricultural irrigation) water sources [50,62,63]. The extreme arid conditions of the Mojave and Colorado deserts results in an irregular distribution of mosquito larval habitats with some areas, such as irrigated valleys and the Salton Sea, supporting large mosquito populations, while other areas support very few. The heterogenous distribution of mosquitoes in the southwestern US may have further restricted the spread of SLEV.

Given the diverse ecologies of the western US, variation in bird and mosquito species distributions may influence viral persistence and dissemination patterns. In central CA and AZ, a single introduction of SLEV was maintained locally year-to-year, while multiple short-lived introductions were observed in southeastern CA. The ecologies of southern AZ and southeastern CA are more similar, as both are arid deserts dotted with cities and smaller towns. Whether SLEV persists in a particular area may be attributable in part to the patchiness of the environment. As for Eastern equine encephalitis virus, another bird-transmitted arbovirus [64], our findings suggest source-sink dynamics in which larger areas with interconnected patches of
suitable host and vector habitat maintain larger and more robust viral metapopulations, while smaller more isolated ecological “islands” (sinks) in the middle of deserts could leave SLEV more vulnerable to stochastic fadeout. Maricopa County, AZ is home to Phoenix, which is surrounded by several irrigated valleys, collectively make for a large area suitable for the hosts and vectors of SLEV. Southeastern CA has smaller urban areas from Palm Springs to Indio and a series of smaller towns on both ends of the Salton Sea in the irrigated Coachella and Imperial Valleys. Other unidentified differences in the local micro-environments or anthropogenic factors, such as vector management strategies or water use, may have contributed to the differences observed between the persistence of SLEV in AZ and the rapid extinction of SLEV lineages introduced into southeastern CA. It is also possible that year-to-year persistence occurred in southern CA but was not detected by our study because sequences were available only from a few well-sampled areas of the desert.

Finally, while the natural dispersal of SLEV-competent birds and mosquitoes seem like the most obvious drivers of SLEV expansion, anthropogenic transport should not be neglected. While humans are considered dead-end hosts for SLEV and cannot perpetuate the SLEV transmission cycle, human behavior could have facilitated the transport of SLEV-infected mosquitoes. Transport and survival of mosquitoes has been reported in personal automobiles [65], as well as during international flights aboard aircraft [35]. Human travel may facilitate the dispersal of SLEV beyond the ecological barriers that influence host and vector movement. For instance, range expansion of SLEV in the Central Valley of CA, an important agricultural region, may have been accelerated by infected mosquitoes hitchhiking in vehicles carrying crops.

In this study, we failed to detect any historically endemic SLEV genotypes (genotypes I, II or V) in the western US after 2014, suggesting that all SLEV activity in the western US is the result of the recent introduction of genotype III. However, it is unclear if historically endemic SLEV genotypes continue to circulate in the remaining portions of the US. Our results also revealed three distinct routes of SLEV dissemination that support the hypothesis that geographic and ecological features, likely the Sierra Nevada, Cascade, and the Transverse Ranges, influenced the movement of SLEV in the western US. It is possible that similar geographic barriers may also influence the movement of other avian arboviruses in the US, like WNV and Western equine encephalitis virus, the latter of which has not been detected in CA since 2006, but which re-emerged in Mexico in 2019 [66]. Understanding natural barriers to virus dissemination may allow public health officials to exploit geographic features affecting arbovirus spread to better protect local communities and to tailor mitigation strategies to areas that are more susceptible to virus migration, such as low-elevation valleys. Taken together, the results of this study highlight the importance of viral genome sequencing in virus surveillance.

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**Author Contributions**

**Conceptualization:** Daniele M. Swetnam, Aaron C. Brault, Lark L. Coffey.

**Data curation:** Daniele M. Swetnam.

**Formal analysis:** Daniele M. Swetnam.

**Funding acquisition:** Lark L. Coffey.

**Investigation:** Jackson B. Stuart.

**Methodology:** Daniele M. Swetnam, Jackson B. Stuart, Payal D. Maharaj, Ying Fang, Sandra Garcia, Christopher M. Barker, Lark L. Coffey.

**Project administration:** Lark L. Coffey.

**Resources:** Kirk Smith, Marvin S. Godsey, Harry M. Savage, Vonnita Barton, Bethany G. Bolling, Nisha Duggal, Aaron C. Brault, Lark L. Coffey.

**Visualization:** Daniele M. Swetnam, Katherine Young.

**Writing – original draft:** Daniele M. Swetnam, Lark L. Coffey.

**Writing – review & editing:** Daniele M. Swetnam, Jackson B. Stuart, Katherine Young, Payal D. Maharaj, Ying Fang, Christopher M. Barker, Kirk Smith, Marvin S. Godsey, Harry M. Savage, Vonnita Barton, Bethany G. Bolling, Nisha Duggal, Aaron C. Brault, Lark L. Coffey.

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