VIROLOGY 218, 343–351 (1996)
ARTICLE NO. 0203

Genetic Conservation of Highlands J Viruses

*Department of Biology, University of California, San Diego, La Jolla, California 92093-0116; and †Center for Tropical Diseases and Department of Pathology, University of Texas Medical Branch, Galveston, Texas 77555-0605

Received November 8, 1995; accepted February 8, 1996

We studied molecular evolution of the mosquito-borne alphavirus Highlands J (HJ) virus by sequencing PCR products generated from 19 strains isolated between 1952 and 1994. Sequences of 1200 nucleotides including portions of the E1 gene and the 3′ untranslated region revealed a relatively slow evolutionary rate estimated at $0.9–1.6 \times 10^{-4}$ substitutions per nucleotide per year. Phylogenetic trees indicated that all HJ viruses descended from a common ancestor and suggested the presence of one dominant lineage in North America. However, two or more minor lineages probably circulated simultaneously for periods of years to a few decades. Strains isolated from a horse suffering encephalitis, and implicated in a recent turkey outbreak, were not phylogenetically distinct from strains isolated in other locations during the same time periods. Our findings are remarkably similar to those we obtained previously for another North American alphavirus, eastern equine encephalomyelitis virus, with which Highlands J shares primary mosquito and avian hosts, geographical distribution, and ecology. These results support the hypotheses that the duration of the transmission season affects arboviral evolutionary rates and vertebrate host mobility influences genetic diversity.

INTRODUCTION

Highlands J (HJ) virus is an arthropod-borne virus in the family Togaviridae, genus *Alphavirus.* HJ virus is grouped within the western equine encephalitis (WEE) antigenic complex, which also includes Sindbis, Fort Morgan, Aura, and WEE viruses (Calisher and Karabatsos, 1988; Calisher *et al.,* 1988). Like other alphaviruses, HJ virus has a single-stranded plus-sense RNA genome of about 12 kilobases. Upon cell infection, genomic RNA is released into the cytoplasm and serves as a template for the viral polymerase to transcribe a minus-strand RNA intermediate. From the minus-strand template, a full-length genomic RNA and subgenomic 26S RNA are synthesized. Genomic RNA is translated as a polyprotein to yield the nonstructural proteins nsP1–4, while the subgenomic 26S RNA, identical to the 3′ one-third of the genome, encodes only the structural proteins (Strauss and Strauss, 1994).

The WEE complex and the eastern equine encephalomyelitis (EEE) and Venezuelan equine encephalomyelitis (VEE) complexes are the major alphavirus serogroups found in the New World. HJ virus was originally categorized as a subtype of WEE virus, but serologic tests and oligonucleotide mapping later indicated that it is a distinct alphavirus species (Calisher *et al.,* 1988; Trent and Grant, 1980). The virus has been isolated only in North America, where it is transmitted among passerine birds in freshwater swamps by the mosquito vector *Culiseta melanura* (Hayes and Wallis, 1977). Enzootic transmission foci occur along the Atlantic seaboard of the United States, and the Gulf Coast as far west as Texas. Inland foci also occur in Michigan and upstate New York. HJ virus is ecologically similar if not identical to EEE virus in North America, sharing geographic distribution, primary mosquito vector, and vertebrate amplifying hosts (Morris, 1988; Scott and Weaver, 1989). However, unlike EEE, HJ virus is not believed to be pathogenic for humans or equines, with the exception of its implication in a 1964 case of encephalitis in a Florida horse (Jennings *et al.,* 1966; Karabatsos *et al.,* 1988). HJ virus was recently recognized as an important poultry pathogen; widespread infection of turkeys was reported in North Carolina during 1991 (Ficken *et al.,* 1993), and HJ virus has been implicated in disease in a variety of other domestic avian species, including pheasants, chukar partridges, ducks, emus, and whooping cranes (Guy *et al.,* 1993, 1994a,b; Wages *et al.,* 1993).

Recent studies of New World alphaviruses indicate a high degree of genetic conservation and slower rates of evolution than many non-arthropod-borne viruses (Weaver, 1995; Weaver *et al.,* 1992b, 1994). EEE virus appears to evolve more slowly in North America than in the tropics (Weaver *et al.,* 1994). This suggests that the amount of virus replication, related to the duration of the transmission season and temperature in temperate vs tropical locations, may be one factor regulating arbovirus evolutionary rates. This hypothesis predicts that other alphaviruses restricted to temperate regions, such as HJ virus, should evolve at rates comparable to North

[1] Present address: Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92717.
[2] To whom reprint requests should be addressed.

0042-6822/96 $18.00

TABLE 1

Highlands J Viruses Sequenced

Strain	Code	Year of isolation	Place of isolation	Host	Passage history[a]
L2-34A	LA52	1952	Louisiana	Mosquito	ce3
B230	FL60	1960	Florida	Bird	p5, sm2
WX3-2AP	TX63	1963	Texas	Bird	sm1
64A-1519	FL64	1964	Florida	Horse	p4, sm1
NCJ5-6X	NC65	1965	North Carolina	Mosquito	de1
MW8-5AD	MD68	1968	Maryland	Mosquito	p2, sm1
SP72-666	CT72	1972	Connecticut	Mosquito	p2
73V2540	MA73	1973	Massachusetts	Mosquito	p2, sm1
B-8-74	CT74	1974	Connecticut	Bird	C6/36-1
78-3331	MA78	1978	Massachusetts	Mosquito	C6/36-1
WC-431	NJ81	1981	New Jersey	Bird	C6/36-1
83-230	MA83	1983	Massachusetts	Mosquito	Unpassaged
111-84	CT84	1984	Connecticut	Mosquito	sm2
3-81690	RI90	1990	Rhode Island	Mosquito	v1
R362	NC92	1992	North Carolina	Turkey	v1
F25	RI92	1992	Rhode Island	Mosquito	v1
93-504	MA93	1993	Massachusetts	Mosquito	Unpassaged
RV94-204	NJ94	1994	New Jersey	Mosquito	ce1, de1
M32	RI94	1994	Rhode Island	Mosquito	v1

[a] C6/36, mosquito cell; ce, chick cell; de, duck embryo cell; p, undetermined passage; sm, suckling mouse; v, Vero cell.

American EEE virus, estimated at 1.6×10^{-4} substitutions per nucleotide per year. The similarity of the HJ and EEE transmission cycles also predicts that patterns of virus dispersal (gene flow), influencing genetic diversity, should be comparable. To test these hypotheses, we sequenced PCR products from representative HJ virus strains isolated from 1952 to 1994. Rates and patterns of evolution estimated from phylogenetic analyses were similar those reported previously for EEE virus, supporting these hypotheses.

MATERIALS AND METHODS

Virus preparation and PCR amplification

The 19 HJ virus strains we analyzed are listed in Table 1. Virus stocks were prepared on BHK-21 cell culture monolayers at 37° with a multiplicity of infection of 0.1 to 1.0 PFU per cell. After cytopathic effects were evident, viral RNA was extracted from 100 μl of the cell culture supernatant using Trizol (BRL Laboratories), a phenol, guanidine isothiocyanate monophasic solution, according to the manufacturer's protocol. Two micrograms each of tRNA and glycogen were added as carriers during precipitation. Following centrifugation, the precipitated RNA pellet was washed with 70% ethanol, dried in vacuum, and resuspended in 1 μl RNasin ribonuclease inhibitor (Promega) and 9 μl 5 m*M* Tris, 0.1 m*M* EDTA (pH 8.0) buffer. The RNA was mixed with 100 ng of an antisense primer of sequence 5′-GAAATTTTAAAAACA-AAATA-3′ designed to anneal to the 3′ end of the HJ genome adjacent to the poly(A) tail, according to the previously published 3′ sequence (Ou *et al.,* 1983). The RNA–primer mixture was heated to 65° for 1 min, followed by gradual cooling to 23°. cDNA was synthesized using 200 units of Superscript reverse transcriptase (BRL) according to the manufacturer's protocol, with incubation at 38° for 30 min. For PCR amplification, 300 ng each of the anti-sense primer and a sense primer of sequence 5′-TACCCNTTYATGTGGGGW-3′, designed to anneal to a conserved alphavirus genome region homologous to nucleotides 10247–10264 of EEE virus (Weaver *et al.,* 1993), were added. The reaction volume was increased to 100 μl with the addition of PCR buffer (10 m*M* KCl, 10 m*M* $(NH_4)_2SO_4$, 20 m*M* Tris (pH 8.8), 2 m*M* $MgSO_4$, 0.1% Triton X-100, 0.1 mg/ml BSA) and 1.25 U *Taq* polymerase (Stratagene). This PCR mix was placed in a thermal cycler for 30 amplification cycles as follows: heat-denaturation at 95° for 30 sec, primer annealing at 44° for 30 sec, and extension at 72° for 2 min.

DNA sequencing and analyses

Direct dideoxynucleotide sequencing of PCR products was performed using the Cyclist Exo(−) Pfu DNA Sequencing kit (Stratagene) according to the manufacturer's protocol or an Applied Biosystems 377 automated sequencer using the Prism Dyedeoxy chemistry. The 11 DNA primers used are listed in Table 2. The nucleotide sequences for 19 strains of HJ virus were aligned with homologous sequences of o'nyong-nyong (Levinson *et al.,* 1990), Ross River (Faragher *et al.,* 1988), Semliki Forest (Garoff *et al.,* 1980), chikungunya (M. D. Parker, unpublished, GenBank Accession No. L37661), Sindbis (Strauss *et al.,* 1984), EEE (Weaver *et al.,* 1993), WEE

TABLE 2

Oligonucleotide Primers Used for Sequencing

Sequence (5′–3′)	Genetic sense
TACCCNTTYATGTGGGGW	Plus
ATCGGTAAAGAGCATTCACG	Plus
GGAGCATCATCCCTC	Plus
CAACGATGTCTGTGGAATC	Minus
AACTCATTTCGAGCACGGT	Minus
AGAGGAGTGCGAATGGACAG	Minus
GATAATCATAGAGCTGCAGG	Minus
TTATGCACCACGCTTCCT	Minus
GAAATTTTAAAAACAAAATA	Minus

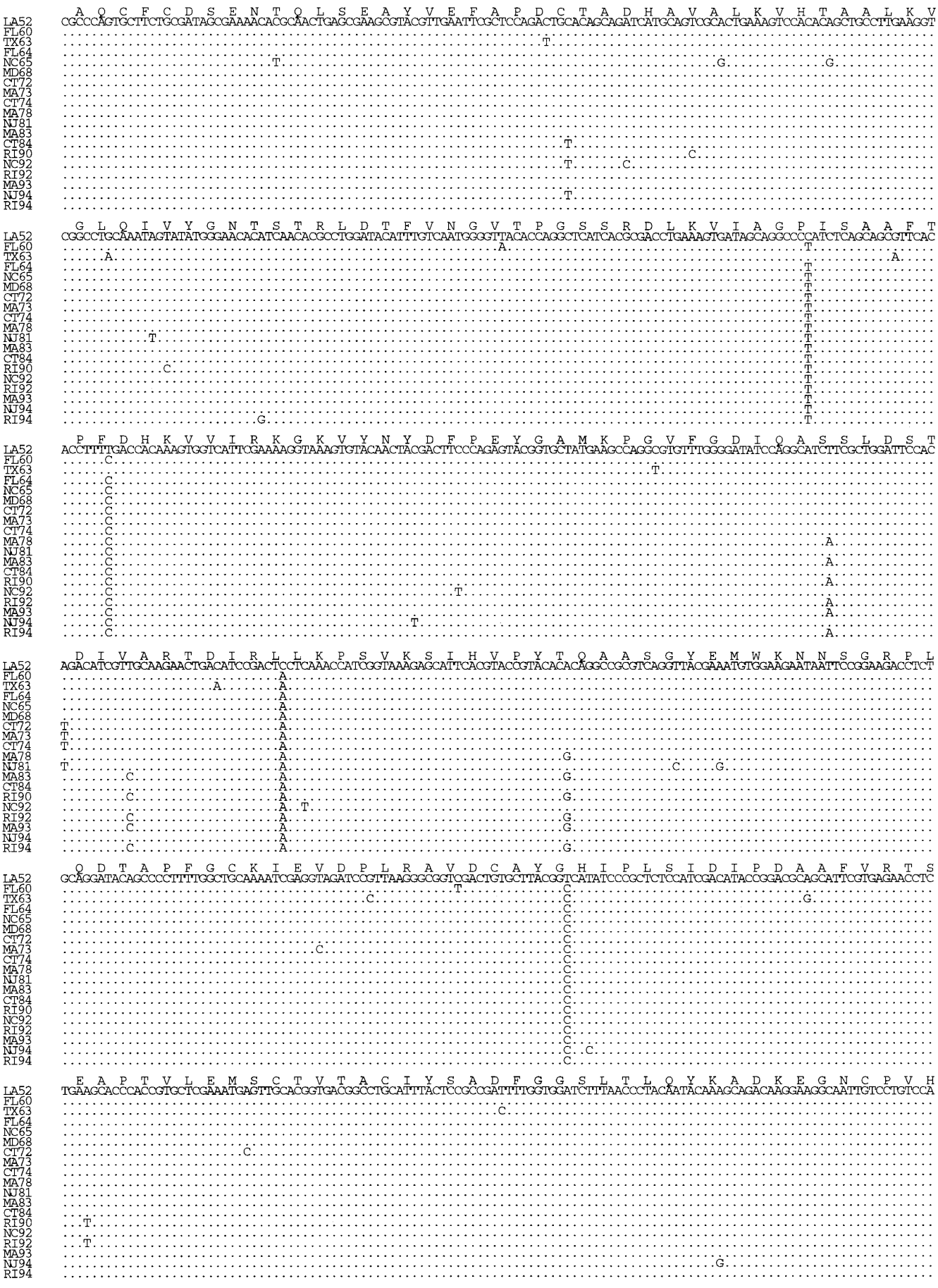
(Hahn *et al.,* 1988), Middelburg (Strauss *et al.,* 1983), Aura (Rumenapf *et al.,* 1995), and VEE antigenic subtypes IAB (Kinney *et al.,* 1986), IC, ID (Kinney *et al.,* 1992), IE (Sneider *et al.,* 1993), and II (Sneider *et al.,* 1993) viruses using the PILEUP program of the Genetics Computer Group (Devereux *et al.,* 1984). The complete homologous sequence of WEE virus was included in the phylogenetic analyses, while only E1 nucleotides homologous to positions 10267–11271 of the EEE virus genome (Weaver *et al.,* 1993) were included for the other alphaviruses because of uncertainty in alignments in the C-terminal end of E1 and the 3′ untranslated region. Aligned sequences underwent phylogenetic analysis using the PAUP parsimony program (Swofford, 1991) and the NEIGHBOR neighbor-joining program implemented in the PHYLIP package (Felsenstein, 1993). Parsimony analysis was implemented using the heuristic algorithm with unordered characters, and sequences were added at random with 100 replications. The ACCTRANS option was used for parsimony character state optimization. The one-parameter formula of Jukes and Cantor (1969) was used to generate the distance matrix for neighbor-joining analysis. Bootstrap analyses with 200 resamplings were used to place confidence values on groupings within trees. The MacClade program (Maddison and Maddison, 1992) was used to trace character changes within parsimony trees.

RESULTS

Aligned sequences of the 19 strains of HJ virus, 1200 nucleotides in length, are presented in Fig. 1. The sequences were remarkably conserved, with maximum nucleotide divergence of 2.2% and E1 amino acid divergence of 1.7%. Among alphaviruses, HJ sequences were most similar to that of WEE virus (ca. 78% nucleotide identity, 89% E1 amino acid identity) and least similar to those of o'nyong-nyong and chikungunya viruses (ca. 50% nucleotide identity, 42% E1 amino acid identity). Parsimony analysis of HJ and homologous alphavirus sequences yielded one tree of minimum (2671 steps) length. The neighbor-joining tree showed identical relationships. A parsimony tree depicting relationships among the different alphaviruses examined is presented in Fig. 2. All HJ viruses formed a monophyletic group supported by a bootstrap value of 100%, indicating that they descended from a common ancestor. All other relationships among alphaviruses were consistent with our previous phylogenetic studies (Rico-Hesse *et al.,* 1995; Weaver *et al.,* 1992a, 1993, 1994). As indicated by serological relationships (Calisher *et al.,* 1988), the closest relatives of HJ virus were other members of the WEE antigenic complex; most closely related was WEE, followed by the Old World WEE complex members Sindbis and Ockelbo viruses, and the New World Aura virus.

A parsimony tree showing relationships among the 19 HJ virus isolates is presented in Fig. 3. Within the HJ group, relationships partially but not fully reflected the year of isolation of the strains examined. For example, the basal two strains were isolated in 1963 and 1952, respectively, while the most recent isolates were found in terminal branches. This topology, as well as the overall genetic conservation we observed, indicated that a single, predominant lineage of HJ virus existed during the past 4 decades in most regions sampled. However, the basal position of the 1963 Texas isolate, followed by the 1952 Louisiana strain, suggested that two or more independent lineages occurred during the earlier time period. Forcing the TX63 strain into the remaining group (leaving LA52 in the basal position) increased the tree length by two or more steps. Because no other HJ virus isolates were available from Texas, we were unable to determine if or how long a distinct lineage or genotype may have persisted there. Some other terminal groupings, such as CT84-NC92-NJ94, CT72-MA73-CT74-NJ81, and MA78-MA83-MA93-RI90-RI92-RI94, which excluded other isolates from the same time period, also suggested simultaneous circulation multiple HJ virus lineages for limited time periods. The nearly consistent grouping of isolates from Massachusetts and Rhode Island, as well as New Jersey and Connecticut (Fig. 3), indicated that some lineages may have been regionally defined and independent for periods of a decade or more. However, our limited collection of viruses precluded determining with certainty whether cocirculating lineages were geographically restricted. These patterns of limited genetic diversity are very similar to those we reported previously for EEE virus (Weaver *et al.,* 1994).

HJ virus strain 64A-1519 (Fig. 3; FL64), isolated from the brain of a Florida horse suffering from encephalitis in 1964 (Jennings *et al.,* 1966; Karabatsos *et al.,* 1988), was genetically similar to other isolates from the 1960s (Fig. 3). Strain R362, isolated during a North Carolina turkey outbreak in 1992, grouped with strains from Connecticut and New Jersey isolated during the past decade. Based on the genome portion analyzed, our phylogenetic analysis yielded no evidence that HJ viruses responsible for equine or poultry disease belong to a genotype dis-


A Q C F C D S E N T Q L S E A Y V E F A P D C T A D H A V A L K V H T A A L K V
LA52 CGCCCAGTGCTTCTGCGATAGCGAAAACACGCAACTGAGCGAAGCGTACGTTGAATTCGCTCCAGACTGCACAGCAGATCATGCAGTCGCACTGAAAGTCCACACAGCTGCCTTGAAGGT
FL60
TX63
FL64
NC65
MD68
CT72
MA73
CT74
MA78
NJ81
MA83
CT84
RI90
NC92
RI92
MA93
NJ94
RI94
G L Q I V Y G N T S T R L D T F V N G V T P G S S R D L K V I A G P I S A A F T
LA52 CGGCCTGCAAATAGTATATGGGAACACATCAACACGCCTGGATACATTTGTCAATGGGGTTACACCAGGCTCATCACGCGACCTGAAAGTGATAGCAGGCCCCATCTCAGCAGCGTTCAC
FL60
TX63
FL64
NC65
MD68
CT72
MA73
CT74
MA78
NJ81
MA83
CT84
RI90
NC92
RI92
MA93
NJ94
RI94
P F D H K V V I R K G K V Y N Y D F P E Y G A M K P G V F G D I Q A S S L D S T
LA52 ACCTTTTGACCACAAAGTGGTCATTCGAAAAGGTAAAGTGTACAACTACGACTTCCCAGAGTACGGTGCTATGAAGCCAGGCGTGTTTGGGGATATCCAGGCATCTTCGCTGGATTCCAC
FL60
TX63
FL64
NC65
MD68
CT72
MA73
CT74
MA78
NJ81
MA83
CT84
RI90
NC92
RI92
MA93
NJ94
RI94
D I V A R T D I R L L K P S V K S I H V P Y T Q A A S G Y E M W K N N S G R P L
LA52 AGACATCGTTGCAAGAACTGACATCCGACTCCTCAAACCATCGGTAAAGAGCATTCACGTACCGTACACACAGGCCGCGTCAGGTTACGAAATGTGGAAGAATAATTCCGGAAGACCTCT
FL60
TX63
FL64
NC65
MD68
CT72
MA73
CT74
MA78
NJ81
MA83
CT84
RI90
NC92
RI92
MA93
NJ94
RI94
Q D T A P F G C K I E V D P L R A V D C A Y G H I P L S I D I P D A A F V R T S
LA52 GCAGGATACAGCCCCTTTTGGCTGCAAAATCGAGGTAGATCCGTTAAGGGCGGTCGACTGTGCTTACGGTCATATCCCGCTCTCCATCGACATACCGGACGCAGCATTCGTGAGAACCTC
FL60
TX63
FL64
NC65
MD68
CT72
MA73
CT74
MA78
NJ81
MA83
CT84
RI90
NC92
RI92
MA93
NJ94
RI94
E A P T V L E M S C T V T A C I Y S A D F G G S L T L Q Y K A D K E G N C P V H
LA52 TGAAGCACCCACCGTGCTCGAAATGAGTTGCACGGTGACGGCCTGCATTTACTCCGCCGATTTTGGTGGATCTTTAACCCTACAATACAAAGCAGACAAGGAAGGCAATTGTCCTGTCCA
FL60
TX63
FL64
NC65
MD68
CT72
MA73
CT74
MA78
NJ81
MA83
CT84
RI90
NC92
RI92
MA93
NJ94
RI94

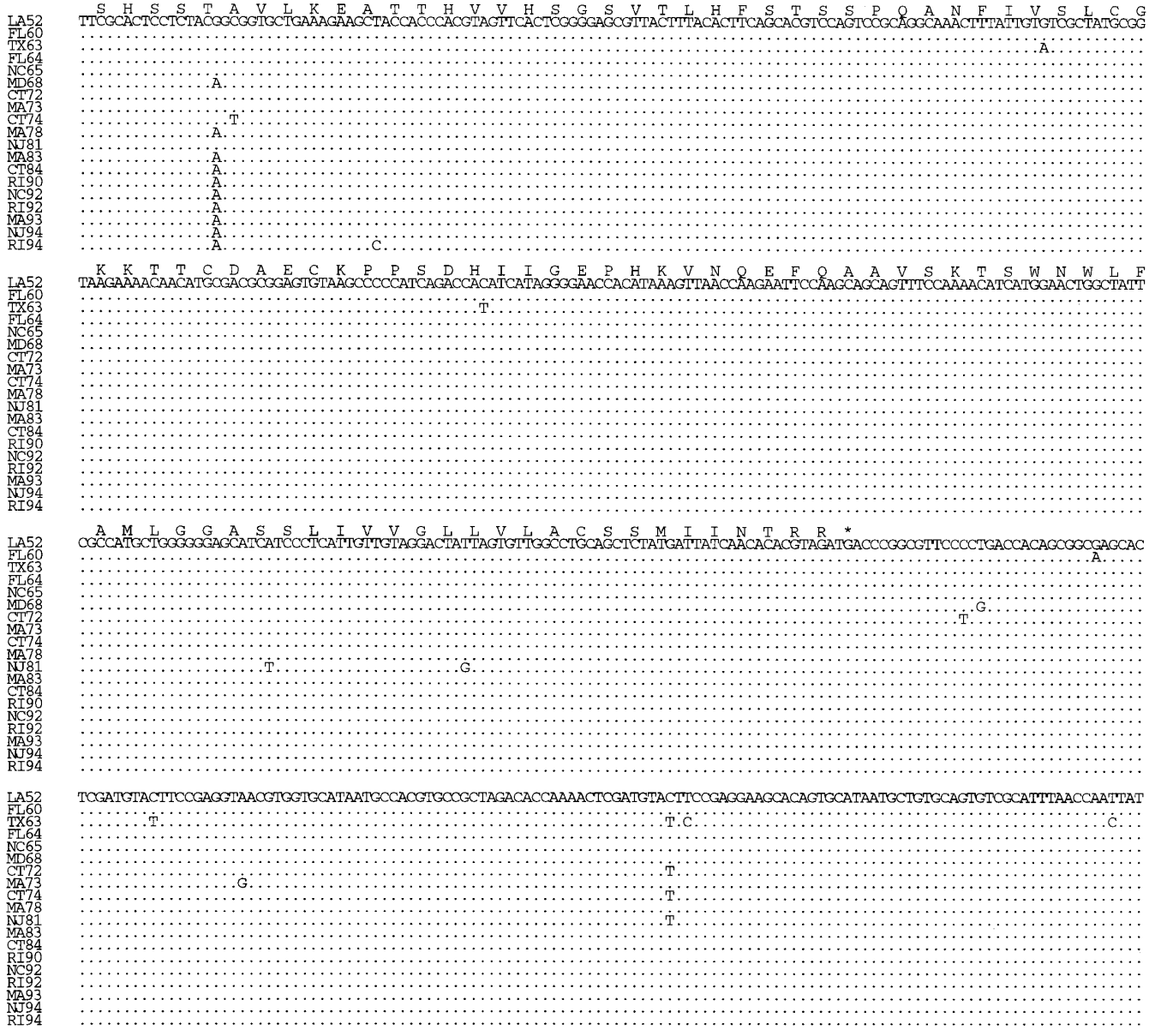


FIG. 1. Aligned nucleotide sequences for 19 strains of Highlands J virus described in Table 1. Amino acid abbreviations are listed above the second position of codons for the E1 coding region.

tinct from that of viruses circulating in other regions of North America during the same time period.

Nucleotide substitutions accompanying HJ virus evolution were examined by comparing ancestral sequences, predicted by the parsimony analysis, with terminal taxa. The HJ clade defined by the hypothetical ancestor at node A (Fig. 3), composed of all isolates except the 1963 Texas strain, was analyzed. This clade was selected because all predicted ancestral sequences (internal nodes) within this clade, as well as nucleotide substitutions represented in its branches, were unambiguous (the node representing the ancestor of the entire HJ virus clade had several ambiguities representing alternative character reconstructions). Of the 39 changes we analyzed, 28 were transitions and 11 were transversions, yielding a ratio of 2.5:1. Within the E1 coding region, 26/34 substitutions were third codon position, 2 were first position, and 6 were second position.

We also examined nucleotide substitutions maintained in the dominant lineage by categorizing changes that distinguished the hypothetical ancestral sequence of this HJ group from those of all strains isolated during the 1990s. Of 16 such substitutions, 13 were transitions (ratio of 4.3:1) and all were third codon position, synonymous substitutions within the E1 coding region. These data indicate strong selection for conservation of the E1 amino

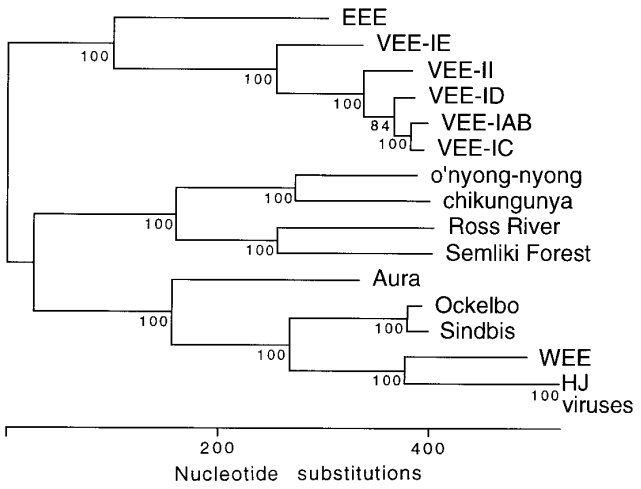


FIG. 2. Parsimony tree of alphaviruses. All Highlands J virus strains fell into a monophyletic group shown in greater detail in Fig. 3. Numbers indicate bootstrap values of 50% or greater for groups to the right in the tree.

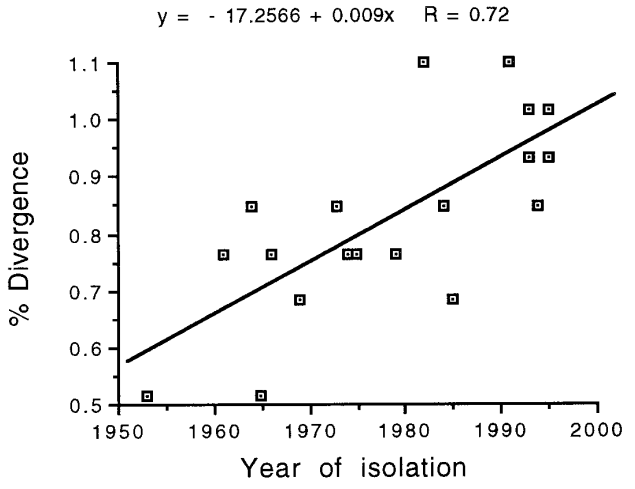


FIG. 4. Regression analysis of the average rate of evolution (nucleotide substitution) for Highlands J viruses isolated from 1952 to 1994. Branch lengths separating each isolate from the hypothetical ancestor of the HJ group, expressed as % divergence, were plotted vs year of isolation. The slope, 0.009%/year, is an estimate of the average rate of evolution.

acid sequence. Although the 3′ untranslated region comprised 13% of the sequence region examined, none of the substitutions that occurred in this portion of the genome were maintained in the dominant lineage. This dearth of substitutions within the 3′ untranslated region may reflect selection for conservation of primary or secondary RNA structures involved in alphavirus genome replication (Strauss and Strauss, 1994).

We estimated the rate of nucleotide substitution within the HJ virus genome portion examined using the regression method described previously (Buonagurio *et al.,* 1986; Weaver *et al.,* 1994). Each isolate within the HJ group was plotted by year of isolation and percent divergence vs the hypothetical ancestor of the entire HJ clade (Fig. 4). The regression yielded an estimated evolutionary rate of 0.009%/year or 9×10^{-5} substitutions/nucleotide/year. If multiple HJ virus lineages were cocirculating during this time period, this rate would represent an average for those lineages. The data indicated no obvious change in the evolutionary rate during the 1952–1994 time period examined. We also estimated the evolutionary rate by totaling substitutions (branch lengths) between the most recent (RI94, NJ94) isolates and node A (Fig. 3). Node A was selected over the ancestor of the entire HJ group because the branch separating it from the LA52 isolate was relatively short (two steps), suggesting that it occurred not long before 1952. The total branch lengths separating this node from the 1990s isolates were 7 and 8 substitutions, respectively, or 0.58 and 0.67% divergence. Assuming that the ancestor occurred in 1952 (the year of isolation for the basal isolate in this group), we obtained an estimated rate of 0.014–0.016%/year or $1.4–1.6 \times 10^{-4}$ substitutions/nucleotide/year. This rate must be considered a maximum estimate because the hypothetical ancestor may have occurred before 1952.

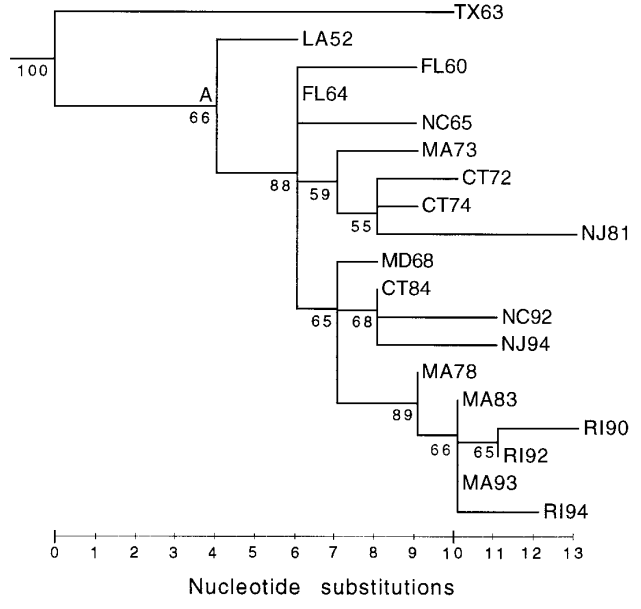


FIG. 3. Phylogenetic tree showing relationships among 19 strains of HJ virus. Numbers indicate bootstrap values of 50% or greater for groups to the right in the tree. Node A indicates the hypothetical ancestor of all of the viruses except the 1963 Texas isolate. Codes for viruses are defined in Table 1.

DISCUSSION

Evolutionary rate

Our results underscore the genetic stability and sequence homogeneity of HJ viruses. Because the nonstructural genes of alphaviruses are more highly conserved than the structural genes (Strauss and Strauss, 1994), the genomic evolutionary rate may be lower than

our estimate for the E1/3′UTR region. The majority of substitutions we observed within the E1 gene were synonymous, consistent with strong selection for the current amino acid sequence of the E1 envelope glycoprotein. These indications of strong selection support the hypothesis that arbovirus evolution is constrained by the dual selective pressures imposed by obligate alternation between vertebrate and invertebrate hosts (Weaver *et al.,* 1992b).

Relatively slow evolutionary rates in the range 1.6–5 $\times$ 10^{-4} substitutions/nucleotide/year have been estimated for several other New World alphaviruses including EEE, WEE, and VEE. Our estimated HJ substitution rate of 0.9–1.6 $\times$ 10^{-4} substitutions/nucleotide/year is similar to that of EEE virus in North America (Weaver *et al.,* 1994), but lower than those for EEE, VEE, and WEE viruses that circulate in South and Central America. This supports the hypothesis that the abbreviated alphavirus transmission season and lower temperatures in temperate regions slow annual rates of alphavirus evolution (Weaver, 1995; Weaver *et al.,* 1992b). The lack of appreciable change in the rate of HJ virus evolution from 1952 to 1994 is inconsistent with the hypothesis that declines in passerine bird populations are responsible for the changes in EEE virus evolutionary rates we reported previously (Weaver *et al.,* 1994).

Genetic diversity

We obtained evidence that two or more distinct, cocirculating genotypes of HJ virus were maintained for several years or a few decades. However, the virus appears to evolve over longer time frames as a single, predominant lineage. This genetic conservation and low diversity are consistent with studies of other North American alphaviruses including EEE (Weaver *et al.,* 1994) and WEE (Trent and Grant, 1980). In contrast, EEE (Weaver *et al.,* 1994) and VEE viruses (Weaver *et al.,* 1992b) are more diverse in South and Central America.

Possible factors influencing the diversity of alphaviruses have been reviewed previously (Weaver, 1995; Weaver *et al.,* 1992b). One hypothesis, that the diversity of vector taxa influences arbovirus diversity, is not entirely supported by accumulating data. EEE and HJ viruses are transmitted by mosquitoes in the genus *Culiseta,* a species-poor taxon in North America. However, WEE virus, which also displays low diversity and genetic conservation (Trent and Grant, 1980), utilizes the more diverse genera *Culex* and *Aedes* (Aviles *et al.,* 1992; Reisen and Monath, 1988). Another hypothesis, that host mobility influences arbovirus diversity, is better supported. Whereas the most diverse New World alphaviruses, VEE and South American variety EEE viruses, utilize rodent hosts in tropical locations, less diverse viruses such as North American EEE, WEE, and HJ primarily infect birds. Birds are presumably more efficient than rodents in disseminating viruses among isolated transmission foci. Efficient virus dissemination may constrain alphavirus evolution by enhancing genome exchange (gene flow), effectively minimizing founder effects and genetic drift (Weaver, 1995; Weaver *et al.,* 1992b). Dissemination may also enhance competition among cocirculating genotypes, facilitating competitive exclusion.

HJ virus maintenance

The source of HJ and EEE viruses that initiate the annual transmission cycle in temperate locations of North America is unknown. Transovarial transmission (TOT) has been reported for the alphaviruses Ross River (Kay, 1982), WEE (Fulhorst *et al.,* 1994), and Sindbis (Dhileepan *et al.,* 1996). However, TOT of EEE and HJ viruses has not been demonstrated experimentally (Sprance, 1981) or supported by consistent field isolation of virus from male mosquitoes or larvae (Hayes and Wallis, 1977; Morris, 1988). Alternatively, these viruses could overwinter within resident birds that become persistently infected in the summer or fall and become or remain viremic during the following spring. Although field studies of EEE virus provide circumstantial evidence for this hypothesis (Crans *et al.,* 1994), experimental infections are needed to determine if birds become persistently infected with EEE and/or HJ viruses. The isolation of WEE virus from experimentally infected birds up to 10 months after inoculation suggests that the chronic avian infections could serve as an alphavirus overwintering mechanism (Reeves *et al.,* 1958).

A third hypothesis for transmission initiation, the annual reintroduction of viruses by migrating birds from subtropical foci, is not supported by the regional basis of some northeastern groupings in our HJ virus trees; these groupings suggest virus maintenance in temperate foci throughout the year, as was also indicated for EEE virus (Weaver *et al.,* 1994). However, our limited sampling of viruses precludes ruling out the more complex explanation that HJ virus is annually reintroduced from subtropical locations in a geographically specific manner.

Migratory birds are less likely to be infected with EEE virus than summer and permanent residents of New Jersey (Crans *et al.,* 1994). This finding supports the hypothesis that movement of EEE and HJ viruses among geographic regions, allowing for competitive displacement of cocirculating genotypes, occurs only occasionally. If HJ virus is maintained in temperate transmission foci through the winter, limited dispersal could be provided by postnesting avian wandering, as was suggested for EEE virus (Weaver *et al.,* 1994). This kind of limited dispersal and genetic mixing of HJ viruses may account for the small amount of genetic diversity we observed. More detailed phylogenetic studies, examining more isolates

sampled throughout the geographic range of these viruses, are needed to further delineate these patterns of alphavirus dispersal and maintenance.

ACKNOWLEDGMENTS

We are grateful to Charles Calisher, Yvonne Gonzalez Chase, James Guy, Nick Karabatsos, Robert Shope, Wayne Pizzuti, Barbara Taylor, Robert Tesh, and Dennis Trent for supplying HJ virus isolates. John Holland provided valuable advice with the research and manuscript, and Estelle Bussey provided excellent technical assistance. This work was supported by Grants AI 10984 and AI14627 from the NIH.

REFERENCES

Aviles, G., Sabattini, M. S., and Mitchell, C. J. (1992). Transmission of western equine encephalomyelitis virus by Argentine *Aedes albifasciatus* (Diptera: Culicidae). *J. Med. Entomol.* 29, 850–853.

Buonagurio, D. A., Nakada, S., Parvin, J. D., Krystal, M., Palese, P., and Fitch, W. M. (1986). Evolution of human influenza A viruses over 50 years: Rapid, uniform rate of change in NS gene. *Science* 232, 980–982.

Calisher, C. H., and Karabatsos, N. (1988). Arbovirus serogroups: Definition and geographic distribution. *In* "The Arboviruses: Epidemiology and Ecology" (T. P. Monath, Ed.), Vol. I, pp. 19–57. CRC Press, Boca Raton, FL.

Calisher, C. H., Karabatsos, N., Lazuick, J. S., Monath, T. P., and Wolff, K. L. (1988). Reevaluation of the western equine encephalitis antigenic complex of alphaviruses (family Togaviridae) as determined by neutralization tests. *Am. J. Trop. Med. Hyg.* 38, 447–452.

Crans, W. J., Caccamise, D. F., and McNelly, J. R. (1994). Eastern equine encephalomyelitis virus in relation to the avian community of a coastal cedar swamp. *J. Med. Entomol.* 31, 711–728.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12, 387–395.

Dhileepan, K., Azoulas, J. K., and Gibson, C. A. (1996). Evidence of vertical transmission of Ross River and Sindbis viruses (Togaviridae: Alphavirus) by mosquitoes (Diptera: Culicidae) in southeastern Australia. *J. Med. Entomol.* 33, 180–182.

Faragher, S. G., Meek, A. D. J., Rice, C. M., and Dalgamo, L. (1988). Genome sequences of a mouse-avirulent and a mouse-virulent strain of Ross River virus. *Virology* 163, 509–526.

Felsenstein, J. (1993). "PHYLIP (Phylogeny Inference Package) version 3.5p." Distributed by the author. Department of Genetics, University of Washington, Seattle.

Ficken, M. D., Wages, D. P., Guy, J. S., Quinn, J. A., and Emory, W. H. (1993). High mortality of domestic turkeys associated with Highlands J virus and eastern equine encephalitis virus infections. *Avian Dis.* 37, 585–590.

Fulhorst, C. F., Hardy, J. L., Eldridge, B. F., Presser, S. B., and Reeves, W. C. (1994). Natural vertical transmission of western equine encephalomyelitis virus in mosquitoes. *Science* 263, 676–678.

Garoff, H., Frischauf, A. M., Simons, K., Lehrach, H., and Delius, H. (1980). Nucleotide sequence of cDNA coding for Semlike Forest virus membrane glycoproteins. *Nature* 288, 236–241.

Guy, J. S., Barnes, H. J., Ficken, M. D., Smith, L. G., Emory, W. H., and Wages, D. P. (1994a). Decreased egg production in turkeys experimentally infected with eastern equine encephalitis virus or Highlands J virus. *Avian Dis.* 38, 563–571.

Guy, J. S., Barnes, H. J., and Smith, J. G. (1994b). Experimental infection of young broiler chickens with eastern equine encephalitis virus and Highlands J virus. *Avian Dis.* 38, 572–582.

Guy, J. S., Ficken, M. D., Barnes, H. J., Wages, D. P., and Smith, J. G. (1993). Experimental infection of young turkeys with eastern equine encephalitis virus and highlands J virus. *Avian Dis.* 37, 389–395.

Hahn, C. S., Lustig, S., Strauss, E. G., and Strauss, J. H. (1988). Western equine encephalitis virus is a recombinant virus. *Proc. Natl. Acad. Sci. USA* 85, 5997–6001.

Hayes, C. G., and Wallis, R. C. (1977). Ecology of Western equine encephalomyelitis in the eastern United States. *Adv. Virus Res.* 21, 37–83.

Jennings, W. L., Allen, R. H., and Lewis, A. L. (1966). Western equine encephalomyelitis in a Florida horse. *Am. J. Trop. Med. Hyg.* 15, 96–97.

Jukes, T. H., and Cantor, C. R. (1969). Evolution of protein molecules. *In* "Mammalian Protein Metabolism" (H. N. Munro, Eds.), pp. 21–132. Academic Press, New York.

Karabatsos, N., Lewis, A. L., Calisher, C. H., Hunt, A. R., and Roehrig, J. T. (1988). Identification of Highlands J virus from a Florida horse. *Am. J. Trop. Med. Hyg.* 19, 603–606.

Kay, B. H. (1982). Three modes of transmission of Ross River virus by *Aedes vigilax* (Skuse). *Aust. J. Exp. Biol. Med. Sci.* 60, 339–344.

Kinney, R. M., Johnson, B. J. B., Brown, V. L., and Trent, D. W. (1986). Nucleotide sequence of the 26S mRNA of the virulent Trinidad donkey strain of Venezuelan equine encephalitis virus and deduced sequence of the encoded proteins. *Virology* 152, 400–413.

Kinney, R. M., Tsuchiya, K. R., Sneider, J. M., and Trent, D. W. (1992). Genetic evidence that epizootic Venezuelan equine encephalitis (VEE) viruses may have evolved from enzootic VEE subtype I-D virus. *Virology* 191, 569–580.

Levinson, R. S., Strauss, J. H., and Strauss, E. G. (1990). Complete sequence of the genomic RNA of o'nyong-nyong virus and its use in the construction of alphavirus phylogenetic trees. *Virology* 175, 110–123.

Maddison, W. P., and Maddison, D. R. (1992). "MacClade Version 3." Sinauer Associates, Inc., Sunderland, MA.

Morris, C. D. (1988). Eastern equine encephalomyelitis. *In* "The Arboviruses: Epidemiology and Ecology" (T. P. Monath, Ed.), Vol. III, pp. 1–36. CRC Press, Boca Raton, FL.

Ou, J.-H., Trent, D. W., and Strauss, J. H. (1983). The 3′-non-coding regions of alphavirus RNAs contain repeating sequences. *J. Mol. Biol.* 168, 1–15.

Reeves, W. C., Hutson, G. A., Bellamy, R. E., and Scrivani, R. P. (1958). Chronic latent infections of birds with western equine encephalomyelitis virus. *Proc. Soc. Exp. Med.* 97, 733–736.

Reisen, W. K., and Monath, T. P. (1988). Western equine encephalomyelitis. *In* "The Arboviruses: Epidemiology and Ecology" (T. P. Monath, Ed.), Vol. V, pp. 89–137. CRC Press, Boca Raton, FL.

Rico-Hesse, R., Weaver, S. C., Siger, J. d., Medina, G., and Salas, R. A. (1995). Emergence of a new epidemic/epizootic Venezuelan equine encephalitis virus in South America. *Proc. Natl. Acad. Sci. USA* 92, 5278–5281.

Rumenapf, T., Strauss, E. G., and Strauss, J. H. (1995). Aura virus is a New World representative of Sindbis-like viruses. *Virology* 208, 621–633.

Scott, T. W., and Weaver, S. C. (1989). Eastern equine encephalomyelitis virus: Epidemiology and evolution of mosquito transmission. *Adv. Virus Res.* 37, 277–328.

Sneider, J., Kinney, R., Tsuchiya, K., and Trent, D. (1993). Molecular evidence that epizootic Venezuelan equine encephalitis (VEE) I-AB viruses are not evolutionary derivatives of enzootic VEE subtype I-E or II viruses. *J. Gen. Virol.* 74, 519–523.

Sprance, H. E. (1981). Experimental evidence against the transovarial transmission of eastern equine encephalitis virus in *Culiseta melanura. Mosq. News* 41, 168.

Strauss, E. G., Rice, C. M., and Strauss, J. H. (1983). Sequence coding for the alphavirus nonstructural proteins is interrupted by an opal termination codon. *Proc. Natl. Acad. Sci. USA* 80, 5271–5275.

Strauss, E. G., Rice, C. M., and Strauss, J. H. (1984). Complete nucleotide sequence of the genomic RNA of Sindbis virus. *Virology* 133, 92–110.

Strauss, J. H., and Strauss, E. G. (1994). The alphaviruses: Gene expression, replication, and evolution. *Microbiol. Rev.* 58, 491–562.

Swofford, D. L. (1991). "PAUP: Phylogenetic Analysis Using Parsimony, Version 3.0." Illinois Natural History Survey, Champaign, IL.

Trent, D. W., and Grant, J. A. (1980). A comparison of New World alphaviruses in the western equine encephalomyelitis complex by immunochemical and oligonucleotide fingerprint techniques. *J. Gen. Virol.* 47, 261–282.

Wages, D. P., Ficken, M. D., Guy, J. S., Cummings, T. S., and Jennings, S. R. (1993). Egg-production drop in turkeys associated with alphaviruses: Eastern equine encephalitis virus and Highlands J virus. *Avian Dis.* 37, 1163–1166.

Weaver, S. C. (1995). Evolution of alphaviruses. *In* "Molecular Basis of Virus Evolution" (A. J. Gibbs, C. H. Calisher, and F. Garcia-Arenal, Eds.), pp.501–530. Cambridge Univ. Press, Cambridge, UK.

Weaver, S. C., Bellew, L. A., Hagenbaugh, A., Mallampalli, V., Holland, J. J., and Scott, T. W. (1994). Evolution of alphaviruses in the eastern equine encephalomyelitis complex. *J. Virol.* 68, 158–169.

Weaver, S. C., Bellew, L. A., and Rico-Hesse, R. (1992a). Phylogenetic analysis of alphaviruses in the Venezuelan equine encephalitis complex and identification of the source of epizootic viruses. *Virology* 191, 282–290.

Weaver, S. C., Hagenbaugh, A., Bellew, L. A., Netesov, S. V., Volchkov, V. E., Chang, G.-J. J., Clarke, D. K., Gousset, L., Scott, T. W., Trent, D. W., and Holland, J. J. (1993). A comparison of the nucleotide sequences of eastern and western equine encephalomyelitis viruses with those of other alphaviruses and related RNA viruses. *Virology* 197, 375–390.

Weaver, S. C., Rico-Hesse, R., and Scott, T. W. (1992b). Genetic diversity and slow rates of evolution in New World alphaviruses. *Curr. Top. Microbiol. Immunol.* 176, 99–117.