JOURNAL OF VIROLOGY, Jan. 1997, p. 613–623 Vol. 71, No. 1
0022-538X/97/$04.00+0

Recombinational History and Molecular Evolution of Western Equine Encephalomyelitis Complex Alphaviruses

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Received 22 July 1996/Accepted 27 September 1996

Western equine encephalomyelitis (WEE) virus (*Togaviridae*: *Alphavirus*) was shown previously to have arisen by recombination between eastern equine encephalomyelitis (EEE)- and Sindbis-like viruses (C. S. Hahn, S. Lustig, E. G. Strauss, and J. H. Strauss, Proc. Natl. Acad. Sci. USA 85:5997–6001, 1988). We have now examined the recombinational history and evolution of all viruses belonging to the WEE antigenic complex, including the Buggy Creek, Fort Morgan, Highlands J, Sindbis, Babanki, Ockelbo, Kyzylagach, Whataroa, and Aura viruses, using nucleotide sequences derived from representative strains. Two regions of the genome were examined: sequences of 477 nucleotides from the C terminus of the E1 envelope glycoprotein gene which in WEE virus was derived from the Sindbis-like virus parent, and 517 nucleotide sequences at the C terminus of the nsP4 gene which in WEE virus was derived from the EEE-like virus parent. Trees based on the E1 region indicated that all members of the WEE virus complex comprise a monophyletic group. Most closely related to WEE viruses are other New World members of the complex: the Highlands J, Buggy Creek, and Fort Morgan viruses. More distantly related WEE complex viruses included the Old World Sindbis, Babanki, Ockelbo, Kyzylagach, and Whataroa viruses, as well as the New World Aura virus. Detailed analyses of 38 strains of WEE virus revealed at least 4 major lineages; two were represented by isolates from Argentina, one was from Brazil, and a fourth contained isolates from many locations in South and North America as well as Cuba. Trees based on the nsP4 gene indicated that all New World WEE complex viruses except Aura virus are recombinants derived from EEE- and Sindbis-like virus ancestors. In contrast, the Old World members of the WEE complex, as well as Aura virus, did not appear to have recombinant genomes. Using an evolutionary rate estimate (2.8×10^{-4} substitutions per nucleotide per year) obtained from E1-3′ sequences of WEE viruses, we estimated that the recombination event occurred in the New World 1,300 to 1,900 years ago. This suggests that the alphaviruses originated in the New World a few thousand years ago.

The western equine encephalomyelitis (WEE) complex is an antigenically related group of arthropod-borne viruses in the genus *Alphavirus*, family *Togaviridae*. Six viruses have been assigned to the complex: Aura, Highlands J, Fort Morgan, Buggy Creek, and WEE viruses occur in the New World; Sindbis virus is restricted to the Old World. Strain Y62-33, considered a distinct virus on the basis of previous analyses, was isolated in Russia (3, 5) (Table 1).

Enzootic transmission cycles of WEE complex viruses vary widely as to vector and vertebrate hosts, as well as distribution. WEE virus is transmitted enzootically in western North America among passerine birds by mosquito vectors, primarily *Culex tarsalis*. This virus has also been isolated in South America and Cuba, where transmission cycles are poorly understood. Highlands J virus occurs only in eastern North America, where it is transmitted among passerine birds by the ornithophilic mosquito, *Culiseta melanura*, in a transmission cycle similar if not identical to that of eastern equine encephalomyelitis (EEE) virus (20). Fort Morgan (21) and Buggy Creek (23) viruses are transmitted in western North America among cliff swallows by the bug *Oeciacus vicarius*. Sindbis viruses are also avian viruses transmitted throughout much of the Old World by various ornithophilic mosquitoes (34).

WEE complex viruses also vary widely as pathogens of humans and domestic animals. WEE virus, first isolated in 1930, has been responsible for periodic, extensive equine epizootics and epidemics of encephalitis in North America (37). WEE epizootics have also occurred periodically in the Pampa and Espinal biogeographic zones of northern Argentina (39), where *Aedes albifasciatus* has been incriminated as an epizootic vector (1). Highlands J virus was originally categorized as a subtype of WEE virus, but detailed serologic tests and oligonucleotide mapping indicated that it is a distinct alphavirus species (5, 46). Highlands J virus is not believed to be pathogenic for humans or horses, with the exception of a 1964 case of encephalitis in a horse in Florida (24, 26). However, the virus was recently recognized as an important poultry pathogen; widespread infection of turkeys was reported in North Carolina during 1991 (11), and Highlands J virus has also been implicated in disease in a variety of other domestic avian species, including pheasants, chukar partridges, ducks, emus, and whooping cranes (16–18, 47). The three other New World viruses belonging to this complex, Buggy Creek, Fort Morgan, and Aura viruses, are not known to cause disease in humans or domestic animals. The viruses called Sindbis virus are widely distributed throughout the Old World and exhibit considerable variability in pathogenicity, sometimes causing fever, rash, arthralgia, or polyarthritis. Epidemic outbreaks caused by Sindbis virus have been reported from a variety of locations (34).

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TABLE 1. Classification of WEE antigenic complex alphaviruses[a]

Virus[b]	Subtype	Antigenic variety[b]	Distribution
WEE		Fleming	California
		McMillan (ON41)	Ontario, Canada
		R-43738 (SD83)	South Dakota
		AG80-646 (AR80)	Argentina
		BeAr102091 (BR66)	Brazil
Y62-33 (RU62)			Russia
Highlands J			Eastern North America
Fort Morgan			Western North America
Buggy Creek			Oklahoma
Sindbis	(I) Sindbis		Africa, Asia, Europe, Australia
	(II) Babanki		Africa
	(III) Ockelbo		Europe
	(IV) Whataroa		New Zealand
	(V) Kyzylagach		Azerbaijan
Aura			South America

[a] Information is from Calisher et al. (5).
[b] Codes indicated in parentheses are listed in Table 2.

WEE viruses have been studied in some detail. Serologic analyses using neutralization tests have revealed antigenic diversity; strains McMillan, R-43738, AG80-646, and BeAr102091 are categorized as subtypes or varieties of the Fleming strain (5). Several strains of WEE virus have also been characterized genetically (46) by oligonucleotide fingerprint analyses. All isolates from 1941 to 1975 were remarkably homogeneous, with an estimated 90% or greater nucleotide sequence identity.

Hahn et al. (19) sequenced the 26S structural genome portion of WEE virus strain BFS1703, a 1953 isolate from California. They reported that the capsid amino acid sequence was most like that of EEE virus, while the E1 and E2 envelope glycoproteins were more like those of Sindbis virus. Hahn et al. concluded that this strain of WEE virus had a recombinant genome derived from EEE and Sindbis-like virus parents. Levinson et al. (29) confirmed the structural gene relationships using phylogenetic analyses. Weaver et al. (52) later determined that the nonstructural genes of WEE virus are also EEE virus-like, and placed the recombination event before divergence of the North and South American varieties of EEE virus.

To further elucidate the molecular evolution of WEE complex viruses and the timing of the recombination event that gave rise to WEE virus, we used phylogenetic analyses employing two different regions of the genome to characterize all members of the WEE complex. Our results indicate that all New World members except Aura virus descended from a recombinant ancestor that occurred in the New World ca. 1,300 to 1,900 years ago. Detailed studies of WEE viruses revealed several independent lineages; some appeared to be restricted to South America, while others included representatives from both South and North America.

MATERIALS AND METHODS

Virus preparation and PCR amplification. The alphavirus strains we sequenced are listed in Table 2. Virus stocks were prepared on BHK-21 cell monolayers at 37°C with a multiplicity of infection of 0.1 to 1.0 PFU per cell. After cytopathic effects were evident, RNA was extracted from 100 μl of the supernatant with Trizol (BRL Laboratories), according to the manufacturer's protocol. Two micrograms each of tRNA and glycogen was added as a carrier during precipitation. Following centrifugation, the precipitated RNA pellet was washed with 70% ethanol, dried in a vacuum, and resuspended in 1 μl of RNasin ribonuclease inhibitor (Promega) and 9 μl of 5 mM Tris–0.1 mM EDTA (pH 8.0) buffer. The RNA was mixed with 100 ng of antisense cDNA primers and heated to 65°C for 1 min, followed by gradual cooling to 23°C. cDNA was synthesized by using 200 U of Superscript reverse transcriptase (BRL) according to the manufacturer's protocol and incubated at 38°C for 30 min. For PCR amplification, 300 ng of antisense and sense primers was added. The reaction mixture volume was increased to 100 μl with the addition of PCR buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris [pH 8.8], 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg of bovine serum albumin per ml), and 1.25 U of *Taq* polymerase (Stratagene). This PCR mix was placed on a thermal cycler for 30 amplification cycles as follows: heat denaturation at 95°C, 30 s; primer annealing at 44°C, 30 s; and extension at 72°C, 2 min.

Primers were selected to anneal to conserved sequences in the alphavirus genome (44). For cDNA synthesis, antisense primers of sequences 5′-GAAAT GTTAAAAACAAAATT-3′ and 5′-GAAATTTTAAAAACAAAATA-3′, designed to anneal to the 3′ untranslated regions (3′UTRs) of Sindbis and WEE viruses adjacent to the poly(A) tail, were used for the E1 region. A sense primer of sequence 5′-TACCCNTTYATGTGGGGW-3′, designed to anneal to a conserved alphavirus sequence homologous to nucleotides 10247 to 10264 of EEE virus (52), was added for the PCR. Buggy Creek virus was amplified from RNA extracted from purified virus by using an oligo(dT) cDNA primer. The nsP4 region of all viruses was amplified by using antisense primers of sequence 5′-T TAGGACCACCGTAGAGA-3′, 5′-TTAGGTCAGCCGTAGAGGGT-3′, and 5′-TTAGGTCAGCCGTAGAGGGT-3′ designed to anneal to the 26S junction regions of Sindbis, EEE, and WEE viruses. A sense primer of sequence 5′-GA TGAAATCNGGVATGTT-3′, designed to anneal to a conserved alphavirus sequence (nucleotides 6982 to 6999 of EEE virus [52]), was used for the PCR amplification.

Sequencing and phylogenetic analyses. Most PCR products were cloned into the PCRII vector (Invitrogen) and sequenced by using plasmid-specific primers as well as those used for PCR amplification. WEE virus PCR products for the E1-3′UTR region were sequenced directly by using two sense (5′-AATTGAAG TGGAGCCTCT-3′ and 5′-CGACCACATAATTGGAGAAC-3′) and two antisense (5′-TTATGCACCACGCTTCCT-3′ and 5′-TATTTTGTTTTTAAAATT TC-3′) primers. Dideoxynucleotide sequencing was performed by using the Cyclist Exo(−) Pfu (Stratagene) or Applied Biosystems Prism automated DNA sequencing kit according to the manufacturers' protocols. Whataroa virus clones were generated and sequenced by standard methods described previously (41). The E1-3′UTR nucleotide sequences for 38 strains of WEE virus were aligned with homologous sequences of chikungunya (35a; GenBank Accession no. L37661), o′nyong-nyong (29), Ross River (9), Semliki Forest (14), Sindbis (43), EEE (52), WEE (19), Highlands J (7), Middelburg (42), Aura (38), and Venezuelan equine encephalomyelitis (VEE) antigenic subtypes IAB (27), ID (28), IE (35), and II (35) viruses by using the PILEUP program of the Genetics Computer Group (8). Only E1 nucleotides homologous to positions 10795 to 11271 of the EEE virus genome (52) were included for the alphaviruses other than WEE virus because of uncertainty in alignments in the C-terminal end of E1 and the 3′UTR. Aligned sequences underwent phylogenetic analysis by using the PAUP parsimony program (45) and the NEIGHBOR neighbor-joining program included in the PHYLIP package (10). Parsimony analysis was implemented by using the heuristic algorithm and both ordered (ratio of transition to transversion, 4.5:1, on the basis of previous estimates for EEE and Highlands J viruses [7, 50]) and unordered characters, and sequences were added at random with 100 replications. The one-parameter formula of Jukes and Cantor (25) was used to generate the distance matrix for neighbor-joining analysis. Bootstrap resampling was used to place confidence values on groupings within trees. The MacClade program (30) was used to trace character changes within parsimony trees.

Nucleotide sequence accession numbers. The nucleotide sequences we determined were submitted to the GenBank library under accession numbers U60357 to U60408.

RESULTS

WEE complex sequences from E1. Part of the E1 (C-terminus) envelope glycoprotein gene totaling 477 nucleotides and illustrated in Fig. 1 was sequenced for representatives of all viruses in the WEE complex not previously examined (Table 2; Fig. 2). These sequences were aligned with those of previously sequenced alphaviruses. A tree summarizing the phylogenetic analyses is presented in Fig. 3. All parsimony and distance matrix analyses yielded phylogenetic trees with similar branching patterns. The only difference in the trees was the position of Whataroa virus; it appeared at the base of the WEE-High-

TABLE 2. WEE complex alphaviruses sequenced for phylogenetic analyses

Virus name or code	Strain	Location[a]	Date (yr, mo-yr, or mo-day-yr)	Host	Passage level[b]
Babanki	Y-251	Cameroon	1969	*Mansonia africana*	p?, sm2
Buggy Creek	81V1822	Oklahoma	1981	*Oeciacus vicarius*	de2
EEE-South American	435731	Chepo, Panama	1986	Horse	V2
Highlands J	B230	Florida	1960	Blue jay	p5, sm2
Ft. Morgan	73V1570	Colorado	1973	*Passer domesticus*	wc1, de1, sm1
Kyzylagach	Leiv65A	Azerbaijan	1969	*Culex modestus*	p8, sm1
Whataroa	M78	New Zealand	1962	*Culex pervigilans*	p9, sm1
WEE-CA30	California	San Joaquin Valley, Calif.	7-30	Horse	gp?, sm27
WEE-Fleming	Fleming	California	Unknown	Human	sm5
WEE-GU59	TR25717	Guyana	8-19-59	Horse	p?, sm3
WEE-AR33	Ar Enc MV	Buenos Aires Prov., Argentina	1933	Horse	sm2, V1
WEE-ON41	McMillan	Ontario, Canada	1941	Human	m2, sm2
WEE-CA46	BFS932	Bakersfield, Calif.	1946	*Culex tarsalis*	sm1
WEE-MO50	EP-6	Missouri	1950	Mosquito	ce1
WEE-CA53[c]	BFS1703	Kern Co., Calif.	7-53	*Culex tarsalis*	sm1, C6/36-1
WEE-CA54	BFS2005	Kern Co., Calif.	1954	*Culex tarsalis*	de1
WEE-AR58	CBA87	Cordoba Prov., Argentina	1958	Horse	sm1
WEE-BR61	Rio-1257	Brazil	1961	Horse	p8, wc1, de1, sm
WEE-CA61	A7712	Kern Co., Calif.	3-24-61	*Ammosperus nelsoni*	p5, de1
WEE-RU62	Y62-33	Urdmurt, Russia	1962	*Aedes cantans/cinereus*	sm1
WEE-BR66	BeAr102091	Brazil	1966	*Culex* (*Melanoconion*) *portesi*	sm2, de1
WEE-BR66	BeAn112509	Brazil	1966	Sentinel mouse	p3, sm1
WEE-MT67	Montana 64	Montana	1967	Horse	de1
WEE-CA68	S8 1-22	Paradise, Colo.	8-2-68	*Sciurus griseus*	sm1
WEE-CA71	BFN 3060	Butte Co., Calif.	7-19-71	*Culex tarsalis*	ce1, sm1
WEE-CU71	UPA	Cuba	1971	Unknown	de1, sm1
WEE-OR71	71V1658	Oregon	8-13-71	Horse	p2, sm1
WEE-TX71	TBT-235	Texas	1971	*Gopherus berlandieri*	p1, de1, sm1
WEE-MX72	M2-958	Mexico	1972	*Culex tarsalis*	V2, sm3
WEE-CO72	72V4768	Morgan City, Colo.	7-18-72	*Culex tarsalis*	sm1
WEE-TX73	73V1492	Hale City, Tex.	8-2-73	*Passer domesticus*	p2, sm1
WEE-MN75	75V9291	Wilkin City, Minn.	7-26-75	*Culex tarsalis*	V2
WEE-CA78	BFS 09997	Kern Co., Calif.	6-30-78	*Culex tarsalis*	V1
WEE-AR80	AG80-646	Chaco Prov., Argentina	1980	*Culex* (*Melanoconion*) *ocossa*	V2, sm1
WEE-AR82	AG83-356	Santa Fe Prov., Argentina	1982	*Mansonia* sp.	p2, sm1
WEE-AR83	CBA-CIV26A	Chaco Prov., Argentina	1983	Horse	V2, sm1
WEE-AR83	CBA-CIV76	Santa Fe Prov., Argentina	1983	Horse	V2, sm1
WEE-AR83	CBA-CIV180	Cordoba Prov., Argentina	1983	Horse	V2, sm1
WEE-AR83	CBA-CIV288	Rio Negro Prov., Argentina	1983	Horse	V2, sm1
WEE-CA83	CHLV 53	Riverside Co., Calif.	7-19-83	*Culex tarsalis*	V1
WEE-SD83	R-43738	South Dakota	1983	Human	p1, sm2
WEE-NM85	85-452-NM	New Mexico	1985	*Culex tarsalis*	sm2
WEE-TN87	TN87-3918	Tennessee	1987	*Culex quinquefasciatus*	V1
WEE-CA92	IMPR 441	Imperial Co., Calif.	7-21-92	*Culex tarsalis*	V1
WEE-AZ93	93A-27	Parker, Ariz.	6-9-93	Mosquito	V1

[a] Prov., province; Co., county.
[b] V, Vero cell; m, mouse; sm, suckling mouse; ce, chick embryo cell; de, duck embryo cell; gp, guinea pig; C6/36, mosquito cell; p, unknown passage; wc, wet chicken.
[c] Sequenced previously (19).

lands J-Buggy Creek-Fort Morgan virus group in the parsimony tree generated from unordered characters but appeared at the base of the Kyzylagach-Sindbis-Ockelbo-Babanki virus group when the transition-transversion weighting was used. The character weighting scheme is reported to increase the accuracy of parsimony trees generated from relatively distantly related nucleotide sequences (22), and nsP4 trees (see below) and antigenic relationships (5) both indicate that Whataroa virus belongs to the Sindbis-like virus group. Therefore, placement of Whataroa virus within the Sindbis virus group, as shown in Fig. 3, is probably accurate. Consistent with serological relationships (3, 5), all trees depicted the WEE complex viruses as a monophyletic group descended from a common ancestor. Babanki, Kyzylagach, and Whataroa viruses were closely related to other Sindbis viruses, while Aura virus was more distantly related to this group. The maximum nucleotide sequence divergence within the Sindbis-like virus group was observed between Aura and Kyzylagach viruses, which showed only 57% identity. Buggy Creek and Fort Morgan viruses were very closely related, showing 93% nucleotide sequence identity, and were grouped with Highlands J and WEE viruses.

WEE virus sequences. To allow us to examine the evolution of WEE viruses in greater detail, 37 isolates (Table 2) were analyzed by sequencing the same E1 region used above, as well as the 3′UTR excluding the last 22 nucleotides adjacent to the poly(A) tail, to yield sequences totaling 791 to 865 nucleotides. The sequences generated are presented in Fig. 4. When these sequences were analyzed together with the alphavirus se-

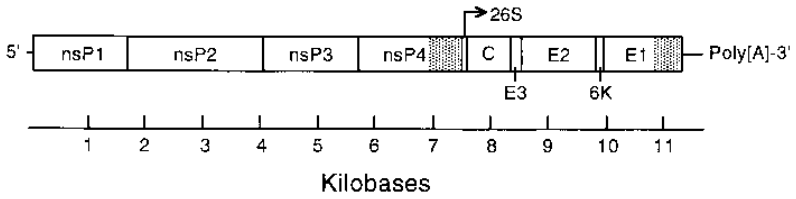

FIG. 1. Genome organization of alphaviruses. Shaded regions of nsP4 and E1 genes show sequence regions used for phylogenetic analyses of WEE complex viruses. The 3′UTR was also used to generate phylogenetic trees of WEE viruses.

quences described above, distance matrix and parsimony (ordered and unordered characters) methods yielded trees with the same branching pattern when zero-length branches were collapsed to yield polytomies. The parsimony tree constructed with unordered characters is shown in Fig. 5. Many of the antigenic subtypes of WEE virus described by Calisher et al. (5) appeared to represent distinct lineages or genotypes of WEE virus. The AR80 strain isolated in northern Argentina from *Culex* (*Melanoconion*) *ocossa* mosquitoes was the most distantly related to the remaining strains examined, followed by another Argentinean (1933, Buenos Aires Province) isolate and a 1966 Brazilian strain. Only single isolates representing most of these lineages were available. However, the lack of closely related isolates from relatively well-sampled North America suggests that these genotypes may be restricted to South America. Isolates from Trinidad and Argentina (1982 and 1983) also appeared to represent lineages distinct from but more closely related to those occurring in North America. The monophyletic nature of isolates from the 1982–1983 Argentinean epizootic indicates that this outbreak probably resulted from a single virus source which spread to several provinces. The distinction between this 1982–1983 epizootic group and all other Argentinean isolates suggests that the source of the outbreak may be an enzootic virus not yet identified or sampled or an epizootic virus that persists in an enzootic transmission cycle. The genetic differences we detected between the AR80, AR58, and AR82-83 strains are consistent with epidemiological (39) as well as mouse neurovirulence and neuroinvasiveness differences (2) reported previously for these viruses.

All of the remaining WEE viruses we sequenced clustered into two closely related groups: group A (Fig. 5) included strains from California, Ontario, Brazil, Mexico, Cuba, and Russia, while group B was composed of viruses isolated from a wide variety of North American locations, as well as Brazil and Argentina. The lack of recent isolates in group A indicates that this lineage may have become extinct after 1972, while the other North American genotype (group B) was still circulating in 1993 (AZ93). In both groups, the oldest isolates occupied basal positions, while the most recent strains occurred near the terminal branches, indicating that these two genotypes evolved overall as single lineages since the 1930s and 1940s. However, smaller, regionally based groupings also were observed, such as the CA53-CA54-CA61-CA78 group from Kern County, California. This group excluded other strains isolated from different locations during the same period, such as MT67, CA68, CA71, OR71, TX73, and MN75. This suggests that multiple, regional WEE virus lineages evolve independently for periods of several years to a few decades. Overwintering of WEE virus within transovarially infected mosquitoes (12) could provide a mechanism for genetic isolation within temperate regions. The presence of both North and South American isolates in groups A and B indicates that some WEE viruses are readily dispersed between the New World continents. This contrasts with other New World alphaviruses, such as EEE and VEE virus, which

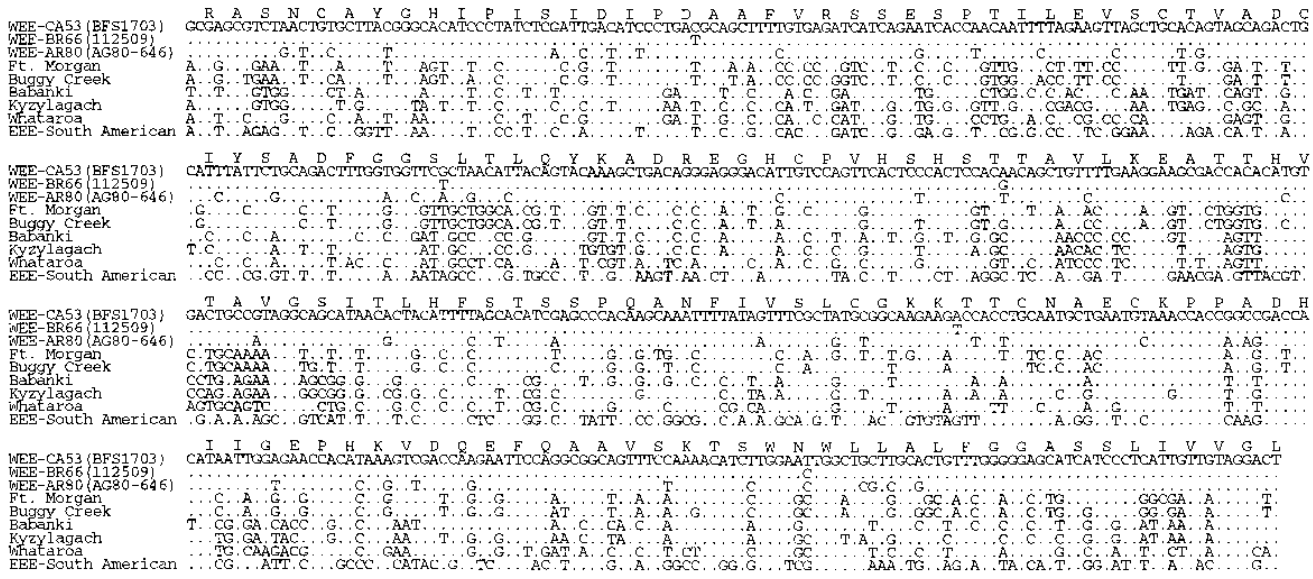

FIG. 2. Aligned nucleotide sequences of WEE complex virus (including the previously published sequence of strain BFS1703) and other alphaviruses, obtained in the present study for the E1 gene region near the C terminus. Amino acids for strain BFS1703 are indicated above the second codon position. Dots indicate that the nucleotide is the same as in the BFS1703 strain.

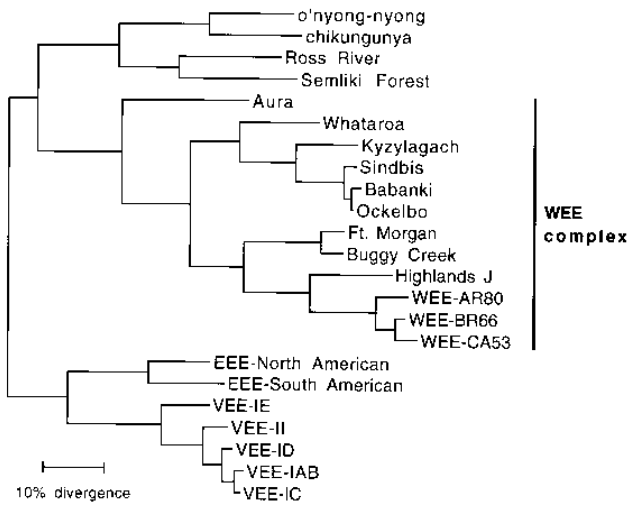

FIG. 3. Phylogenetic tree of representative alphaviruses, derived from the 477 nucleotide sequences shown in Fig. 2 and previously published homologous alphavirus sequences. A bootstrap value of 98% was obtained for the WEE complex group.

include genetically and antigenically distinct viruses restricted to North versus South America.

WEE virus mutations. Nucleotide substitutions accompanying WEE virus evolution were examined by comparing ancestral sequences, predicted by the parsimony analysis, with the sequences we determined. Group B, defined by the hypothetical ancestor at node B (Fig. 5), was analyzed as described previously (7). Of the 60 nucleotide changes represented in the tree, 39 were transitions and 21 were transversions, yielding a ratio of 1.9:1. Within the E1 coding region, 23 of 33 substitutions were third codon position, 6 were first position, and 4 were second position. We also examined nucleotide substitutions maintained in the dominant lineage by categorizing changes that distinguished the hypothetical ancestral sequence of this WEE virus group B from those of strains isolated during the 1990s. Of 17 such substitutions, 14 were transitions (ratio of 4.7:1) and all within the E1 coding region were first- or third-codon-position substitutions. Only one change found in the dominant lineage resulted in an amino acid substitution (threonine to serine). Like the results from detailed studies of Highlands J viruses (7), these data indicate strong selection for conservation of the E1 amino acid sequence.

nsP4 gene analyses. In order to determine if other WEE complex members are recombinants like WEE virus strain BFS1703 (19), we sequenced a second genome region representing the other half of the recombination event. The E1 region described above (Fig. 1) lies within the portion of the WEE virus that is Sindbis virus-like (19). To examine the EEE virus-like portion of all WEE complex viruses, we sequenced a region of nsP4, totaling 517 nucleotides, that is flanked by conserved alphavirus sequences (Fig. 1). The sequences we generated are presented in Fig. 6. All previously published alphavirus sequences (see above) were also included in the phylogenetic analysis. Three WEE virus strains (CA53, AR80, and BR66), representing distinct antigenic subtypes or varieties (5) and appearing as separate lineages in the E1 tree (Fig. 5), were also included. All phylogenetic trees had similar branching patterns, with minor differences using ordered (transversion weighting) characters; a representative tree constructed using unordered characters, and consistent with previous analyses using complete nsP4 sequences (5), is presented in Fig. 7. In contrast to the E1 tree (Fig. 3), the WEE complex viruses did not comprise a monophyletic group in any of the nsP4 trees. Instead, the Sindbis-like viruses, including all Old World members of the WEE virus complex and Aura virus, formed a group distinct from the remaining New World members. Like WEE virus strain BFS1703 (CA53), all WEE viruses, Highlands J, Buggy Creek, and Fort Morgan viruses had nsP4 genes most closely related to those of EEE viruses. Forcing the WEE virus complex group together lengthened all trees by at least 7 steps, indicating that the WEE virus complex nsP4 genes did not evolve exclusively from a common ancestor. The simplest explanation for this result is that the ancestor of the Fort Morgan-Buggy Creek-Highlands J-WEE virus group was a recombinant virus.

WEE virus evolutionary rate. To estimate rates of WEE virus evolution, isolates in the best-sampled group in the WEE virus tree (group B, Fig. 5) were analyzed by regression, with year of isolation plotted versus nucleotide substitutions from their hypothetical ancestor. Because strains falling within this group were very closely related (maximum 2% nucleotide sequence divergence) the probability of sequential substitutions of the same nucleotide was very small, and all mutations should therefore be represented in the data. The regression slope, 0.028% per year (Fig. 8), represented the average rate of sequence chance for this lineage. There was no evidence of appreciable change in this rate from 1946 to 1993. Similar though slightly higher rates of evolution have been reported for EEE (0.043% per year) (50) and VEE (0.05% per year) (53) viruses in South and Central America. The slower rates estimated previously for EEE (0.016% per year) (50) and Highlands J (0.009% per year) (7) viruses in North America may reflect the limited transmission season of alphaviruses restricted to temperate and subtropical locations, resulting in shorter transmission seasons and less genome replication per year (50, 53).

We used this evolutionary rate (0.028% per year) to estimate ancestral divergence events for several WEE virus lineages. The oldest isolate, CA30, was used as a starting point, and internal branch lengths were adjusted for multiple substitutions of the same nucleotides by the one parameter formula (15). We estimated that the AR80 lineage diverged roughly 300 years ago, and this divergence was followed by the AR33 lineage about 180 years ago. Groups A and B, which include both North American and South American isolates, probably diverged around the turn of this century.

Evolutionary history of alphaviruses. Finally, we used information from our phylogenetic studies along with evolutionary rate estimates to generate a hypothesis for the origin of alphaviruses, as well as the time and place of the recombination event that produced the WEE lineage. Invoking parsimony to minimize the number of transoceanic alphaviral introductions, we arrived at the composite tree shown in Fig. 9. Following divergence of Aura virus from the Sindbis virus group, and EEE from VEE virus, recombination between an EEE- and a Sindbis-like virus occurred in the New World roughly 1,300 to 1,900 years ago, on the basis of our evolutionary rate estimated for WEE viruses and branch lengths corrected as described above. A close relative of the Sindbis-like virus progenitor of the Old World WEE complex viruses was later introduced into the Old World. This scenario places the hypothetical ancestor of all extant alphaviruses in the New World. In contrast, alphavirus origination in the Old World would require at least three transoceanic introductions to account for current distributions.

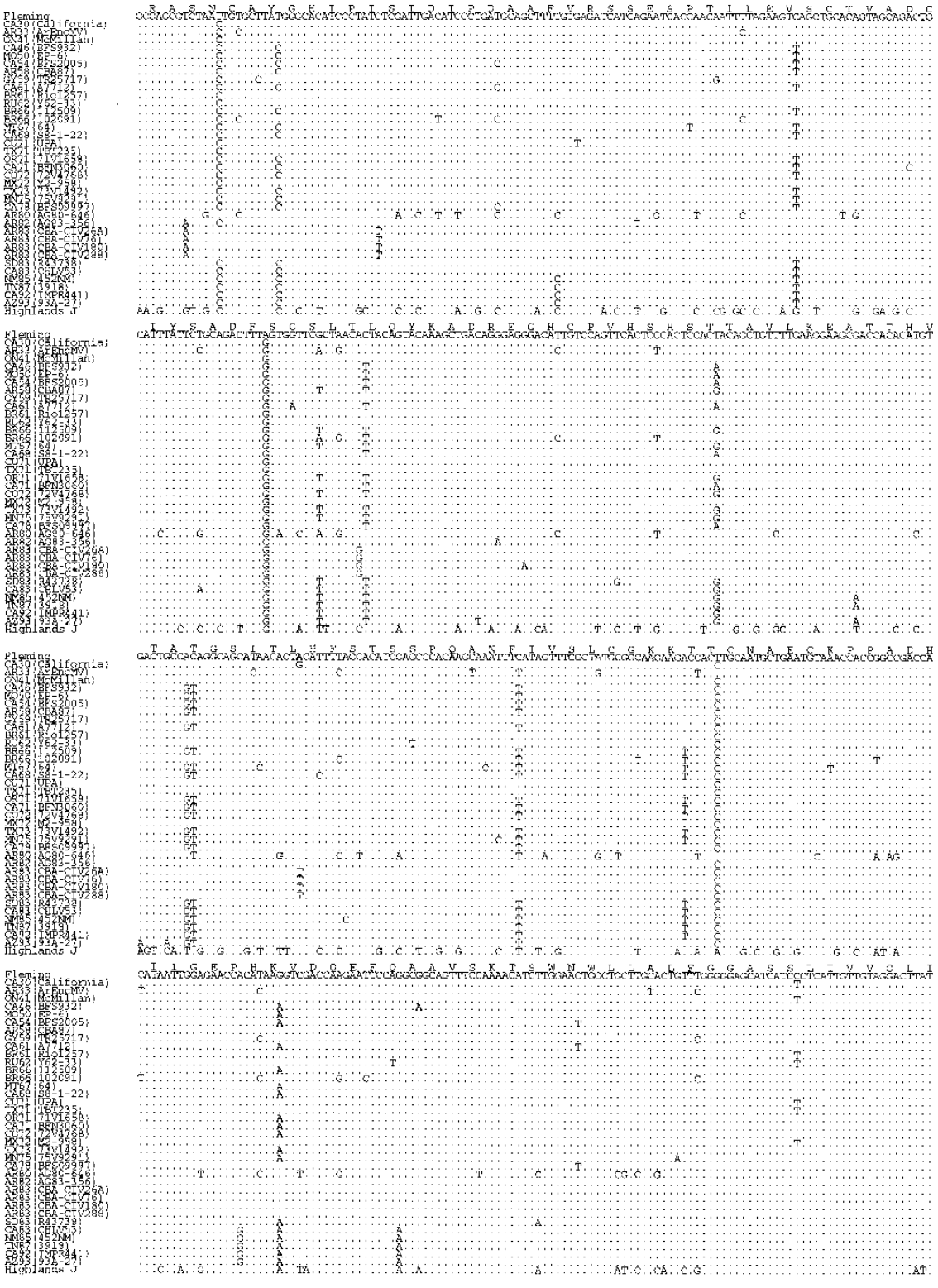

FIG. 4. Aligned nucleotide sequences obtained in the present study for 37 strains of WEE virus, along with the homologous sequence of Highlands J virus, a portion of which was published previously (7). Dots indicate that the nucleotide is the same as that in the Fleming strain; dashes indicate deletions and insertions. Amino acids of the Fleming virus are indicated above the second codon position.

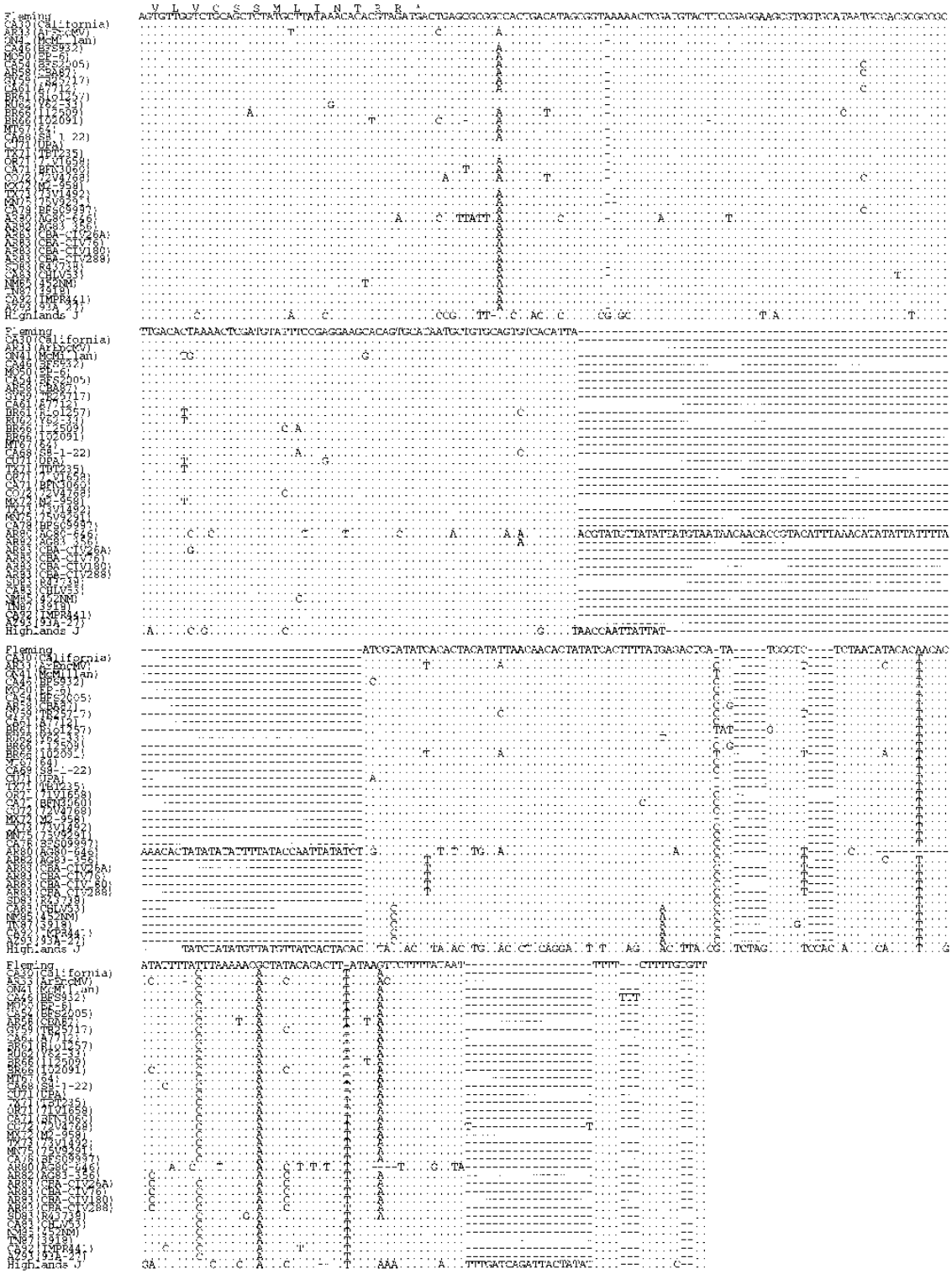

FIG. 4—*Continued.*

DISCUSSION

Evolution and spread of alphaviruses. Phylogenetic trees from two different regions of alphavirus genomes and evolutionary rate estimates for WEE virus and other alphaviruses allow us to hypothesize a scenario for evolution and spread of alphaviruses (Fig. 9). In this model, a single ancestor of all extant alphaviruses occurred in the New World. Previous estimates from complete amino acid sequence comparisons place the initial divergence of this ancestor into the EEE-VEE virus and Aura-Sindbis-Semliki Forest-Ross River virus groups at

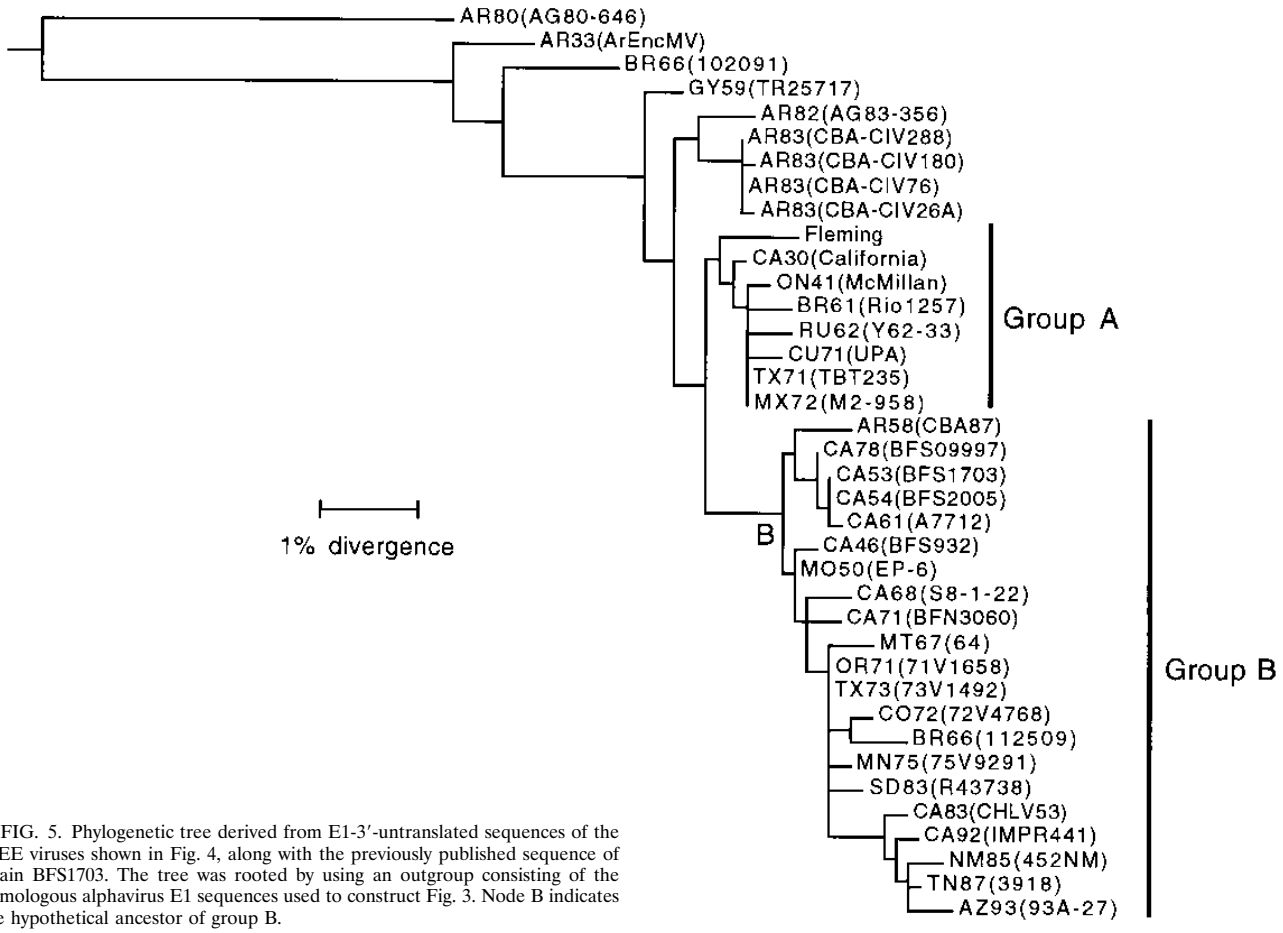

FIG. 5. Phylogenetic tree derived from E1-3′-untranslated sequences of the WEE viruses shown in Fig. 4, along with the previously published sequence of strain BFS1703. The tree was rooted by using an outgroup consisting of the homologous alphavirus E1 sequences used to construct Fig. 3. Node B indicates the hypothetical ancestor of group B.

roughly 3,000 years ago (52). Other ancestral alphavirus lineages could have occurred earlier but become extinct. Homology detected between the replicase components of alphaviruses and a number of plant viruses, most notably tobacco mosaic virus, suggests that the ancestral alphavirus arose by recombination between previously existing but currently unknown viruses that also gave rise to many groups of plant viruses (44). The alphavirus ancestor probably occurred in the tropics, where alphavirus diversity is greatest. The involvement of *Culex* (*Melanoconion*) mosquito vectors and small mammalian vertebrate hosts in tropical enzootic transmission cycles of VEE virus (48), EEE virus (33, 40), and probably WEE virus strain AG80-646 (see below; also reference 6) in the New World suggests that the ancestral alphavirus may have utilized mammalian hosts. However, it is impossible to determine at present whether association with mammals is the ancestral trait and association with birds is derived or vice versa.

Our estimates for the rate of evolution of WEE virus, together with sequence information for WEE, Aura, and Sindbis viruses, place the recombination event between ancestors of EEE and Sindbis-like viruses in the New World tropics 1,300 to 1,900 years ago, after divergence of Aura virus from the ancestor of the Old World Sindbis-like viruses. The host in which this recombination event occurred is unknown, but limited studies of EEE virus populations within natural hosts (49) suggest that dual infections may occur more frequently in vertebrates than in mosquitoes. Our time scale for alphavirus divergence events relies on the assumption that evolutionary rates estimated for this century are representative of the more distant past. Because this assumption is difficult to test, our time estimates for alphavirus evolution should be considered rough approximations. However, the consistency of our estimates for EEE (50), VEE (53), Highlands J (7), and WEE (Fig. 8) virus evolutionary rates, ranging from 0.9×10^{-4} to 5×10^{-4} substitutions per nucleotide per year and 2.8×10^{-4} to 5×10^{-4} for those occurring in the tropics, suggests that these estimates are accurate within an order of magnitude. The WEE virus rate of 2.8×10^{-4} is roughly two to three times that of EEE (50) and Highlands J (7) viruses, consistent with the hypothesis that restriction to temperate and subtropical locations, limiting the transmission season and reducing rounds of genome replication, restricts the evolution of EEE (North American antigenic variety) and Highlands J alphaviruses that occur only in North America (7, 50). Tropical transmission cycles of WEE viruses, all major genotypes of which have been isolated in South America (see Fig. 5), may allow for faster rates of evolution.

Following the alphavirus recombination event that gave rise to the ancestor of WEE virus, a virus closely related to the Sindbis-like virus recombination ancestor was transported to the Old World, where it gave rise to the diverse group of Sindbis-like viruses found in Europe, Africa, Asia, Australia, and New Zealand. An ancestor of the Semliki Forest-Ross River-o′nyong-nyong-chikungunya virus group was also introduced independently into the Old World. Transport of these putative ancestral viruses probably occurred via a migratory

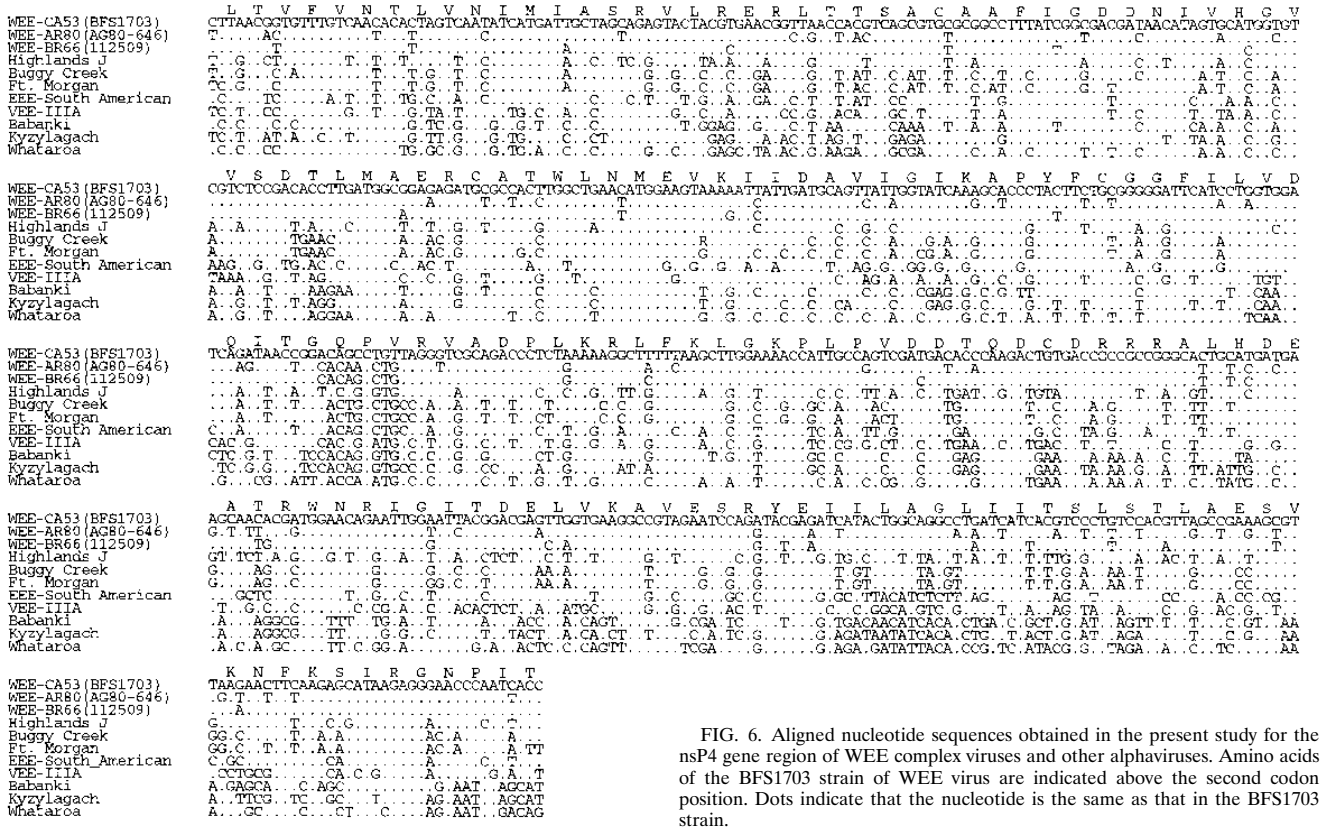

FIG. 6. Aligned nucleotide sequences obtained in the present study for the nsP4 gene region of WEE complex viruses and other alphaviruses. Amino acids of the BFS1703 strain of WEE virus are indicated above the second codon position. Dots indicate that the nucleotide is the same as that in the BFS1703 strain.

bird; Sindbis-like viruses utilize birds as their vertebrate amplifying hosts (34), and latent infection of birds infected with an alphavirus (WEE virus) has been described previously (36).

The recombinant alphavirus apparently remained in the New World, where it diversified to give rise to ancestors of the Buggy Creek-Fort Morgan and WEE-Highlands J virus groups. Our evolutionary rate estimates would place divergence of Buggy Creek virus from Fort Morgan virus ca. 250 years ago; WEE virus diverged an estimated 650 years ago from Highlands J virus and later diversified into several independent lineages (Fig. 5).

WEE virus evolution. Detailed phylogenetic analyses of WEE viruses revealed the presence of multiple lineages or genotypes, most of which have been previously identified as antigenically distinct subtypes or varieties (5). However, strains R43738 and Y62-33 were not phylogenetically distinct from other WEE viruses (Fig. 5). Like an EEE virus antigenic subtype described previously (4, 51), these strains may represent virus lineages that acquired unique amino acid changes in their

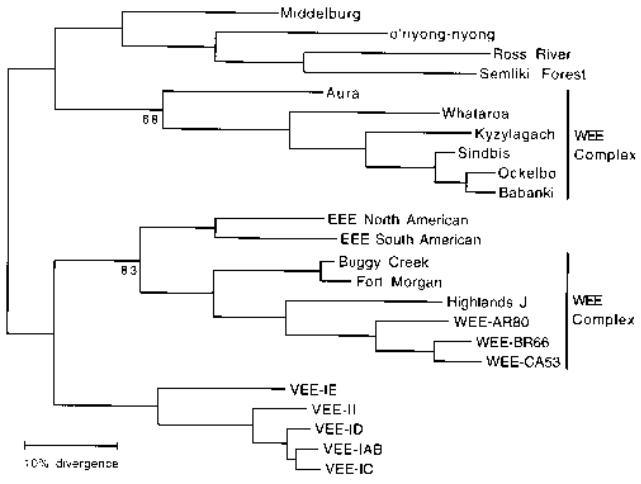

FIG. 7. Phylogenetic tree of representative alphaviruses, derived from nsP4 nucleotide sequences shown in Fig. 6 along with previously published alphavirus sequences. Numbers adjacent to nodes indicate bootstrap values for groups to the right.

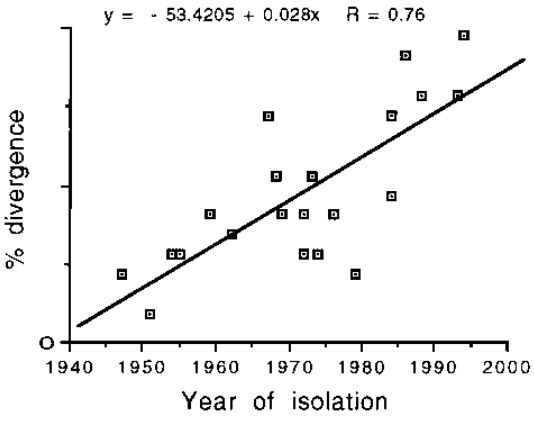

FIG. 8. Regression analysis of average evolutionary rate of WEE viruses in monophyletic group B in Fig. 5. Slope, 0.028x, indicates the average rate of sequence divergence expressed as percentage per year.

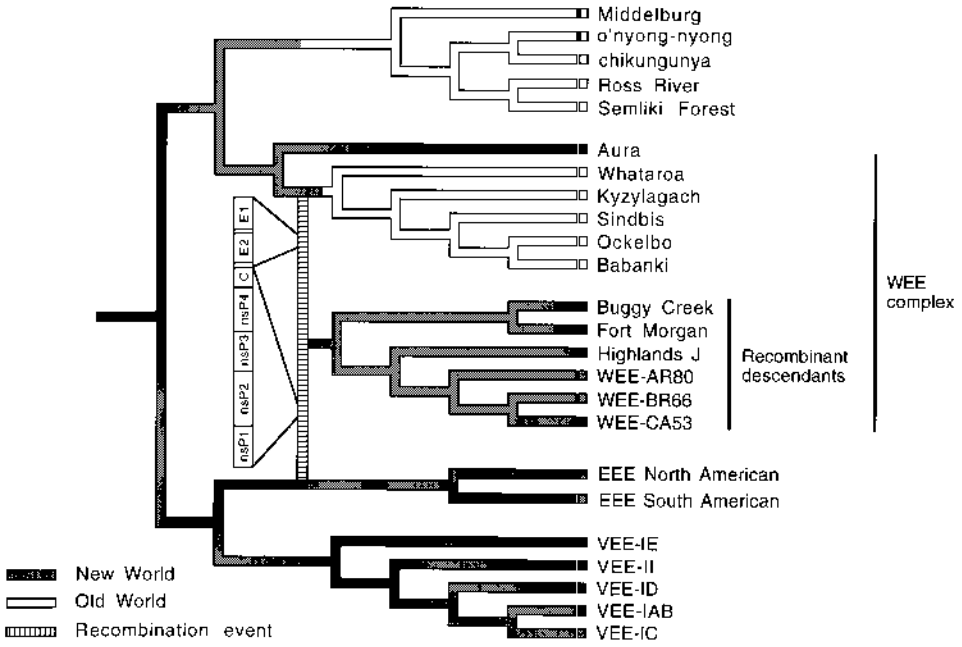

FIG. 9. Phylogenetic tree showing most parsimonious scenario for evolution of alphaviruses. Branch shading indicates presumed locations of hypothetical ancestors and the recombination event between EEE- and Sindbis-like virus progenitors. Branch lengths are uninformative.

antigenic determinants but later became extinct. The similarity of the sequence of strain Y62-33 to that of the McMillan and other North American isolates was confirmed by sequencing PCR products generated from virus stocks obtained from two different reference collections. Further sequencing of antigenic determinants in the E2 glycoprotein would be helpful in determining whether this virus represents a genuine isolate from Russia or a laboratory contaminant that underwent antigenic changes upon passage there.

The WEE virus lineages we refer to as groups A and B in Fig. 5 include strains isolated in both North and South America, whereas other lineages include only isolates from South America. The relatively large number of North American isolates examined suggests that some or all of these South American lineages may be restricted to that continent. The different locations within South America (and within Argentina) represented by the AR33, AR80, AR82-83, BR66, and Trinidad strains also suggest that some of these lineages may be regionally restricted. WEE virus groups A and B comprise one major group of strains more closely related to one another than are the South American genotypes described above. As described above, these groups A and B include isolates from both North and South (Brazil and Argentina) America, suggesting that this genotype is transported readily between the New World continents. This contrasts with the situation for other New World alphaviruses such as EEE and VEE virus, where North and South American viruses are genetically and antigenically distinct (53). We hypothesize that this difference is related to vertebrate host relationships and the mobility of mammals versus avians. Whereas EEE and VEE viruses utilize small mammalian hosts in the tropics, WEE viruses belonging to groups A and B may utilize avian hosts in both North and South America. The ability of mobile, infected avian hosts to disperse alphaviruses may result in continual mixing of gene pools and thus limit diversification, as exhibited by the highly conserved EEE (50) and Highlands J (7) viruses in North America. The more restricted distribution of WEE virus lineages represented by the AR33, AR80, AR82-83, BR66, and Trinidad strains suggests that some of these viruses may utilize vertebrate hosts with limited mobility, such as small mammals. Although vertebrate hosts have not been identified definitively for WEE viruses in South America, Mitchell et al. (31) speculated that mammals, possibly rabbits, may be involved in transmission cycles within the Santa Fe and Rio Negro provinces of Argentina. In that region, most mosquitoes from which WEE virus has been isolated feed principally on mammals, and WEE virus and antibodies were rare in birds but common in small mammals, including rice rats (*Oryzomys* sp.) (32) and introduced European hares (31). *C.* (*M.*) *ocossa*, from which the AG80-646 and two other antigenically related strains of WEE virus were isolated in semitropical Chaco Province of Argentina, also serves as the enzootic vector of VEE variety ID viruses in Panama (13). This association of the AR80 and similar strains of WEE virus with a vector known to maintain another alphavirus within rodent populations suggests that this WEE genotype may also utilize rodent hosts for its maintenance transmission cycle.

ACKNOWLEDGMENTS

We thank Charles Calisher, Ronald Cheshier, Robert Chiles, James Hardy, Nick Karabatsos, Craig Levy, Robert Shope, and Robert Tesh for providing alphavirus strains. Gabriela Aviles, Charles Calisher, Carl Mitchell, and Marta Sabattini made many helpful suggestions during preparation of the manuscript.

The research was supported by grants AI 10984, AI 39508, AI 20612, and AI 10793 from the National Institutes of Health and by the John Sealy Memorial Endowment Fund for Biomedical Research.

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