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Genetic Heterogeneity among Isolates of Ross River Virus from Different Geographical Regions

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The RNase T_1 maps of 80 isolates of Ross River virus from different regions of mainland Australia and the Pacific Islands were compared. Four different clusters of isolates with greater than an estimated 5 to 6% diversity at the nucleotide level were found. There was a pattern of differences between eastern and western Australian strains; however, the pattern was disturbed by overlaps and incursants. Pacific Islands isolates belonged to the eastern Australian topotype. Our findings suggest that certain genetic types of Ross River virus predominate in different geographical regions. In contrast, populations of other important Australian arboviruses (Murray Valley encephalitis, Kunjin, and Sindbis viruses) are distributed across the Australian continent as minor variants of one strain. Our data also show that in one region, strains of Ross River virus with identical RNase T_1 maps circulate during both years when epidemics occur and years when they do not. This finding suggests that Ross River virus epidemics are not dependent on the introduction or evolution of new strains of the virus. Two strains, belonging to the eastern Australian topotype, were isolated in Western Australia. It is likely that viremic humans or possibly domestic livestock travelling by aircraft were responsible for this movement.

Ross River (RR) virus, a mosquito-borne alphavirus, is an etiological agent of epidemic polyarthritis in humans (10, 11, 28). The disease is characterized by arthritic pain, particularly in the peripheral joints, rash, fever, and myalgia (30). RR virus is endemic to Australia. Cases of epidemic polyarthritis are reported from all states of mainland Australia in most years (23). Epidemic polyarthritis due to infection with RR virus has also been reported from the Solomon Islands (38) and from Papua New Guinea (35). During 1979 and 1980, massive outbreaks involving tens of thousands of people occurred in the Fiji Islands (2), American Samoa (39), the Cook Islands (34), and Futuna and Wallis islands and New Caledonia (15); however, little or no RR virus activity has been reported from these islands since then (28).

RR virus has been isolated from a wide range of mosquitoes, although the main vectors in Australia are thought to be *Aedes vigilax*, *Culex annulirostris*, and *A. camptorhynchus* mosquitoes (6, 24, 27). Similarly, most studies show that a wide range of nonmigratory, terrestrial animals, particularly marsupials, are the most likely vertebrate reservoirs or amplifiers of RR virus (reviewed in references 23 and 28). In contrast, the Australian flaviviruses Murray Valley encephalitis (MVE) virus and Kunjin (KUN) virus and the alphavirus Sindbis (SIN) virus are thought to have native and migratory waterbirds as their major vertebrate hosts. The distribution of minor variants of a single type of each of these three viruses across the entire Australian continent (3, 8, 9, 17) suggests the introduction of virus from regions where infections are endemic to regions where they are epidemic.

The more sedentary vertebrate hosts of RR virus may allow microevolution of geographically separated isolates. Indeed, isolates of this virus from different geographical regions have been shown to vary in the ability to kill infant mice (18, 36, 37) and to possess different responses in kinetic hemagglutination and complement fixation tests (47). Woodroofe and coworkers concluded that RR virus isolates were enzootic to their respective regions and probably evolved, in isolation, from a common ancestral virus (47).

Studies of RR virus at the molecular level have been less conclusive. Faragher and coworkers demonstrated extensive variation on the basis of restriction digest profiles of cDNA with a limited number of RR virus isolates (13). They identified three genetic types, each of which contained two subtypes, but the types could not be linked to the source (host or vector) or to their geographical origin. Faragher and coworkers also showed that only one of the genetic types was responsible for the RR virus outbreak in the Pacific Islands. Extensive variability was found in the 3′ untranslated sequences of RR virus field isolates belonging to three of Faragher's genetic types (12). However, the variability was just as marked between isolates from the same region as between those from different regions.

A comparison of the entire sequences of the genomic RNA of two isolates of RR virus obtained 10 years apart from ecologically and climatically different regions of Australia (strain T48 from North Queensland and strain NB5092 from the central coast of New South Wales) revealed remarkably little sequence divergence at the amino acid level (14). During the Pacific Islands outbreak, the virus was found to have been genetically stable (4). This stability was attributed to selective pressures imposed upon the virus during transmission cycles rather than on the genetic stability of the virus (4), shown to be similar to that of non-arthropod-borne RNA viruses (44).

RNase T_1-resistant oligonucleotide mapping (RNase T_1 mapping) has been used to study the genetic relatedness and epidemiology of isolates of several arboviruses, including St. Louis encephalitis (43), dengue 2 (42), Japanese encephalitis (21), and KUN (17) viruses. The aim of this study was to use RNase T_1 mapping to examine the genetic relatedness of multiple isolates of RR virus from different geographical regions and to determine whether (i) the virus survives in

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discrete, geographically separate foci or can move from one region to another, (ii) certain strains are more likely to be associated with epidemics, (iii) an association exists between strains of RR virus and particular vectors or vertebrate hosts, and (iv) the virus evolves within geographical regions.

MATERIALS AND METHODS

Virus strains. When possible, we have attempted to use isolates obtained from field material (either mosquitoes, animals, or humans) at different points in time from each of several different regions of Australia. This was done so that relationships between isolates from one region over time and between isolates from different regions at one point in time could be examined. Details of the 80 RR virus isolates used in this study, including dates of collection, material from which they were isolated, collection site and, where available, passage histories, are shown in Table 1. Prior to producing the RNase T_1 oligonucleotide maps, we showed that all isolates were RR virus by using a tissue culture neutralization assay.

Cell culture and virus stocks. All RR virus isolates were amplified by a single passage in Vero cells grown in medium 199 (GIBCO, Grand Island, N.Y.) supplemented with 2% fetal bovine serum (CSL, Melbourne, Victoria, Australia) and 0.2 g of L-Glu (Sigma, St. Louis, Mo.) per liter. Vero cell monolayers were inoculated at low multiplicity of infection (<0.1), and the infected supernatant fluid was collected, clarified by low-speed centrifugation, aliquoted, and stored at −80°C.

RNase T_1 oligonucleotide mapping and analysis. The method used has been described elsewhere (7). Briefly, virus was grown in Vero cells in 600-cm^2 glass roller bottles (Bellco Glass, Vineland, N.J.). After clarification of the cell culture supernatant, the virus was purified by polyethylene glycol 6000 (BDH, Kilsyth, Victoria, Australia) precipitation and sucrose gradient isopycnic centrifugation. The resultant virus band was pelleted, and virion RNA was extracted with phenol (Bethesda Research Laboratories, Gaithersburg, Md.)-chloroform (BDH) and precipitated under ethanol (BDH). The RNA was rehydrated in 5 μl of deionized diethylpyrocarbonate (Sigma)-treated water and then digested for 1 h with 5 U of RNase T_1 (Calbiochem, La Jolla, Calif.) per μg. The resultant oligonucleotides were 5′ end labelled, using T4 polynucleotide kinase (Amersham International, Amersham, United Kingdom). This mixture was separated by gel electrophoresis in two dimensions, using different conditions for each dimension. The first-dimension separation was at pH 3.5, using 6 M urea (Bio-Rad, Richmond, Calif.) and 10% acrylamide (Bio-Rad), whereas the second-dimension gel contained 22% acrylamide buffered in Tris (Boehringer, Mannheim, Germany)-boric acid (BDH)-EDTA (BDH) (pH 8.3). Autoradiography of the second-dimension gel was carried out with Fuji RX X-ray film (Fuji, Japan) at 4°C for 4 to 16 h.

Pairwise comparisons of fingerprint maps were done manually. Jaccard's algorithm (22) was then used to produce a dissimilarity matrix from which a phenogram was constructed by the unweighted pair group moving average (UPGMA) method. The program for producing the phenograms was from the PHYLIP package (version 3.4) (16).

RESULTS

We produced RNase T_1-resistant oligonucleotide maps for 80 different isolates of RR virus from Australia and the South Pacific. The pairwise comparisons revealed four distinct patterns of the large oligonucleotides. It was not possible to align these patterns. We assume therefore that the divergence between these four groups at the nucleotide level is greater than about 8 to 10% (48) and for convenience have referred to each group as a topotype (genotype associated with a geographical region [43]). Two of the four topotypes were represented by single isolates (SW2191 and K1503); the other two topotypes comprised 27 and 51 members and are referred to as the WK20 and T48 topotypes, respectively. On average, 120 spots, representing about 10% of the viral genome, were present in each map and included in the analysis. No spot was present in all isolates of any topotype. Because of differences in the contrast range between X-ray film and emulsions used for photographs, not all spots that were visible on the X-ray films are evident on the photographs.

Computer analysis of a known full-length sequence of RR virus, deposited in GenBank (RRVNBCG), revealed that the genome contains 126 oligonucleotides equal to or greater than 11 nucleotides in length. The distribution of these oligonucleotides was fairly even across the entire genome (Fig. 1). The analysis also revealed the existence of a 45-mer near the 3′ terminus. This large oligonucleotide can be seen in the autoradiographs shown in Fig. 2A and B but not in Fig. 2C or D. Since no oligonucleotides of a similar size were seen in the latter two maps, we infer that a nucleotide substitution has occurred, resulting in an extra guanine within the 45-base region.

Representative pictures of fingerprints of isolates from each of the four topotypes are shown in Fig. 2. The polyadenylate tail was not always visible but can be seen on the fingerprint of isolate K1503 (Fig. 2C). The distance between isolates within each topotype varied from almost 0 to about 0.21 for the WK20 topotype and about 0 to 0.4 for the T48 topotype. This equates to maximum divergences at the nucleotide level of 1.0 to 2.6% and 2.5 to 5.0%, respectively (1). Analysis of the distance matrices by using the PHYLIP package (NEIGHBOR, UPGMA method; DRAWGRAM) produced phenograms for the WK20 (Fig. 3) and T48 (Fig. 4) topotypes.

There were two major clusters within the WK20 topotype (clusters A and B; Fig. 3) which linked at the lowest level in the tree (distance of 0.23, equivalent to about 1.2 to 3.5% nucleotide divergence). The cluster names (A and B; Fig. 3) do not refer to the same level of dissimilarity as clusters A, B, and C in Fig. 4; they are used for descriptive purposes only. Each of these clusters (Fig. 3) contained isolates from the southwest of Western Australia, the Kimberley region of Western Australia, and the Northern Territory (Fig. 5). This finding suggests the circulation of two somewhat distinct genetic types within this large geographical region.

The time of isolation of viruses belonging to the WK20 topotype ranged from 1977 to 1990. As can be seen from Fig. 3, the clustering did not align with host of origin. It is interesting to note that a human isolate (SHLS2173) obtained during the 1988-to-1989 epidemic in the southwest of Western Australia was most homologous to a mosquito isolate (SW876) from the same region obtained during an interepidemic period some 17 months earlier.

T48, the prototype strain of RR virus, was isolated from mosquitoes collected in 1959. All other isolates in the T48 topotype were obtained between 1969 and 1990. There were three major clusters within this topotype (clusters A, B, and C; Fig. 4). The highest level at which these clusters linked was at a distance of 0.42, which equates to between 2.5 and

TABLE 1. Details of Ross River virus isolates used

No.	Isolate name or no.	Location	Date collected	Source (vector or host)	Passage history[a]	Supplied by[b]:
1	SW876	Lake Clifton, southwest Western Australia	19/8/87	*Aedes camptorhynchus*	2 × C6/36, 2 × Vero	UWA
2	SW877	Lake Clifton, southwest Western Australia	19/8/87	*A. camptorhynchus*	2 × C6/36, 1 × Vero	UWA
3	SW878	Lake Clifton, southwest Western Australia	19/8/87	*A. camptorhynchus*	2 × C6/36, 1 × Vero	UWA
4	SW2089	Mandurah, southwest Western Australia	9/11/88	*Anopheles annulipes*	2 × C6/36, 3 × Vero	UWA
5	SW2191	Australind, southwest Western Australia	9/11/88	*A. camptorhynchus*	4 × C6/36	UWA
6	SW2193	Australind, southwest Western Australia	9/11/88	*A. camptorhynchus*	2 × C6/36, 2 × Vero	UWA
7	SW2197	Australind, southwest Western Australia	9/11/88	*A. camptorhynchus*	2 × C6/36, 2 × Vero	UWA
8	SW2218	Clifton Park, southwest Western Australia	9/11/88	*A. camptorhynchus*	2 × C6/36, 2 × Vero	UWA
9	SW2761	Australind, southwest Western Australia	20/12/88	*A. camptorhynchus*	2 × C6/36, 2 × Vero	UWA
10	SW10330	Clifton Park, southwest Western Australia	23/11/88	*A. camptorhynchus*	1 × C6/36, 1 × Vero	UWA
11	SW10331	Clifton Park, southwest Western Australia	23/11/88	*A. camptorhynchus*	1 × C6/36, 1 × Vero	UWA
12	SHLS2173	Bunbury, southwest Western Australia	11/1/89	Human	1 × C6/36, 3 × Vero	GH
13	SHLS735	Pinjarra, southwest Western Australia	6/1/89	Human	1 × C6/36	GH
14	SW3181	Furnissdale, southwest Western Australia	21/9/89	*A. camptorhynchus*	3 × C6/36, 1 × Vero	UWA
15	SW3183	Furnissdale, southwest Western Australia	21/9/89	*A. camptorhynchus*	3 × C6/36, 1 × Vero	UWA
16	SW3191	Furnissdale, southwest Western Australia	21/9/89	*A. camptorhynchus*	3 × C6/36, 1 × Vero	UWA
17	SW4860	Furnissdale, southwest Western Australia	6/11/89	*A. camptorhynchus*	3 × C6/36, 2 × Vero	UWA
18	SW11747	Mandurah, southwest Western Australia	24/10/90	*A. camptorhynchus*	1 × C6/36, 1 × Vero	UWA
19	SW12181	Mandurah, southwest Western Australia	19/11/90	*A. camptorhynchus*	1 × C6/36, 1 × Vero	UWA
20	SW12358	Australind, southwest Western Australia	5/12/90	*A. camptorhynchus*	1 × C6/36, 1 × Vero	UWA
21	SW12359	Australind, southwest Western Australia	5/12/90	*A. camptorhynchus*	2 × C6/36, 1 × Vero	UWA
22	SW12361	Australind, southwest Western Australia	5/12/90	*A. camptorhynchus*	1 × C6/36, 1 × Vero	UWA
23	WK20	Derby, West Kimberley, Western Australia	March-April 1977	*Culex annulirostris*	? × SMB, 1 × Vero	UWA
24	AN53	Kununurra, East Kimberley, Western Australia	21/3/82	*A. normanensis*	1 × SMB, 1 × Vero	UWA
25	AN205	Smoke Creek, East Kimberley, Western Australia	18/3/82	*A. normanensis*	2 × SMB	UWA
26	AN626	Kununurra, East Kimberley, Western Australia	13/2/83	*A. normanensis*	1 × SMB, 1 × Vero	UWA
27	AN542	Wyndham, East Kimberley, Western Australia	26/1/84	*A. normanensis*	1 × SMB, 1 × Vero	UWA
28	AN561	Kununurra, East Kimberley, Western Australia	8/2/84	*A. normanensis*	2 × SMB	UWA
29	AN572	Kununurra, East Kimberley, Western Australia	13/2/84	*A. normanensis*	2 × SMB	UWA
30	K1503	Wyndham, East Kimberley, Western Australia	26/1/84	*C. annulirostris*	2 × C6/36, 1 × Vero	UWA
31	K1506	Wyndham, East Kimberley, Western Australia	26/1/84	*C. annulirostris*	2 × C6/36, 1 × Vero	UWA
32	K1198	Wyndham, East Kimberley, Western Australia	7/2/84	*C. annulirostris*	2 × C6/36, 1 × Vero	UWA
33	K1205	Wyndham, East Kimberley, Western Australia	7/2/84	*C. annulirostris*	2 × C6/36, 1 × Vero	UWA
34	K1260	Wyndham, East Kimberley, Western Australia	26/1/84	*C. palpalis*	2 × C6/36, 1 × Vero	UWA
35	K1008	Kununurra, East Kimberley, Western Australia	2/3/86	*C. annulirostris*	2 × C6/36, 1 × Vero	UWA
36	V306	Leanyer Dump, Northern Territory	5/1/83	*A. vigilax*	2 × HL, 1 × BHK	MH
37	V309	Jabiru, Northern Territory	2/2/83	*C. annulirostris*	3 × BHK	MH
38	V582	Mataranka, Northern Territory	11/1/84	*A. normanensis*	1 × C6/36, 3 × BHK	MH
39	V583	Larrimah, Northern Territory	11/1/84	*A. normanensis*	1 × C6/36, 3 × BHK	MH
40	V587	Mataranka, Northern Territory	12/1/84	*C. annulirostris*	1 × C6/36, 3 × BHK	MH
41	V993	Casuarina, Northern Territory	14/1/86	*A. vigilax*	1 × C6/36, 3 × BHK	MH
42	T48	Townsville, north Queensland	1959	*A. vigilax*	12 × SMB, 2 × Vero	IDM
43	19575	Charleville, southern central Queensland	11/4/76	*A. normanensis*	1 × SMB, 1 × C6/36	DP
44	19556	Charleville, southern central Queensland	26/5/76	*C. annulirostris*	2 × SMB, 1 × C6/36	DP
45	19502	Charleville, southern central Queensland	19/8/76	*A. normanensis*	2 × SMB, 1 × C6/36	DP
46	19581	Charleville, southern central Queensland	15/9/76	*C. annulirostris*	2 × SMB, 1 × C6/36	DP
47	HA	Rockhampton, central coast, Queensland	1983	Human	2 × C6/36, 2 × SMB	JH
48	CO	Rockhampton, central coast, Queensland	1983	Human	2 × C6/36, 1 × SMB	JH
49	MC	Rockhampton, central coast, Queensland	1983	Human	2 × C6/36, 1 × SMB	JH
50	SE	Cape York Peninsula, far north, Queensland	February 1988	Human	1 × C6/36	DP
51	O1O.385 (RA)	Townsville, north coast, Queensland	1989	Human	1 × C6/36	DP
52	045.573 (GE)	Cairns, north coast, Queensland	1989	Human	1 × C6/36	DP

Continued on following page

TABLE 1—*Continued*

No.	Isolate name or no.	Location	Date collected	Source (vector or host)	Passage history[a]	Supplied by[b]:
53	DR	Cairns, north coast, Queensland	1990	Human	1 × C6/36	DP
54	BE	Winton, western central Queensland	1990	Human	1 × C6/36	DP
55	NB5092	Nelson Bay, central coast, NSW	25/4/69	*A. vigilax*	1 × SMB	IDM
56	NB6024	Nelson Bay, central coast, NSW	21/4/70	*A. vigilax*	? × SMB	IDM
57	96272	Ludwig's Swamp, far south coast, NSW	13/10/82	*Coquillettidia linealis*	2 × SMB, 1 × BHK	MC
58	96614	Wallangat State Forest, near Forster, central coast, NSW	31/10/83	Mixed pool of *A. vigilax*, *A. procax*, and *A. bancroftianus*	1 × BHK	MC
59	97651	Mogo State Forest, south coast, NSW	5/6/86	*A. vigilax*	4 × BHK	MC
60	6142	Mogo State Forest, south coast, NSW	16/3/88	*A. vigilax*	3 × BHK	MC
61	6611	Mogo State Forest, south coast, NSW	7/4/88	*A. vigilax*	3 × BHK	MC
62	2548	Griffith, south central NSW	2/2/89	*C. annulirostris*	1 × C6/36, 3 × Vero	PW
63	1053	Port Stephens (Nelson Bay), central coast, NSW	29/5/89	Mixed pool of *A. procax*, *A. vigilax*, *A. funereus*, and *C. annulirostris*	1 × C6/36, 3 × Vero	PW
64	G25	Gippsland Lakes region, southeast Victoria	December 1988	*A. camptorhynchus*	1 × C6/36, 3 × Vero	JA
65	G26	Gippsland Lakes region, southeast Victoria	December 1988	*A. camptorhynchus*	1 × C6/36, 3 × Vero	JA
66	G88/37	Gippsland Lakes region, southeast Victoria	December 1988	*A. camptorhynchus*	1 × C6/36, 2 × Vero	JA
67	2192	Yarragon, Gippsland, southeast Victoria	26/3/89	Horse (10-year-old mare)	1 × C6/36, 3 × Vero	JA
68	P41472	American Samoa, South Pacific	1979	Human	2 × C6/36	LR
69	F9073	Fiji, South Pacific	1979	Human	Unavailable	JH
70	P41453	Fiji, South Pacific	1979	Human	2 × C6/36	LR
71	218397	Cook Islands, South Pacific	1980	Human	1 × mosquito, 2 × C6/36	LR
72	218072	Cook Islands, South Pacific	1980	Human	1 × mosquito, 2 × C6/36	LR
73	218081	Cook Islands, South Pacific	1980	Human	1 × mosquito, 2 × C6/36	LR
74	218100	Cook Islands, South Pacific	1980	Human	1 × mosquito, 2 × C6/36	LR
75	P42115	Cook Islands, South Pacific	1980	Human	2 × C6/36	LR
76	P42134	Cook Islands, South Pacific	1980	Human	2 × C6/36	LR
77	P42161	Cook Islands, South Pacific	1980	Human	2 × C6/36	LR
78	P42213	Cook Islands, South Pacific	1980	Human	2 × C6/36	LR
79	P42273	Cook Islands, South Pacific	1980	Human	2 × C6/36	LR
80	P41971	Cook Islands, South Pacific	1980	Human	2 × C6/36	LR

[a] History of each isolate at time of arrival in this laboratory. C6/36, C6/36 clone of *A. albopictus* cells; SMB, suckling mouse brain; Vero, Vero (African green monkey kidney) cells; BHK, baby hamster kidney cells; HL, hamster lung cells; mosquito, intrathoracic inoculation of *Toxorhynchites amboinensis* mosquitoes.
[b] UWA, Arbovirus Laboratory, Department of Microbiology, The University of Western Australia; GH, Gerry Harnett, State Health Laboratory Service, Western Australia; MH, Margaret Harmsen, Department of Primary Industry and Fisheries, Northern Territory; DP, Debbie Phillips, Laboratory of Microbiology and Pathology, State Health Laboratory, Department of Health, Queensland; JH, Jenny Haig, Immunology, Department of Medical and Laboratory Science, Queensland University of Technology, Queensland; IDM, Ian Marshall, Department of Biochemistry, Australian National University, Canberra, ACT; MC, Michael Cloonan, Virology Section, Department of Microbiology, The Prince Henry Hospital, New South Wales; PW, Peter Wells, Westmead Medical Entomology Unit, Department of Medicine and Department of Public Health, University of Sydney, New South Wales (NSW); JA, John Aldred, Attwood Veterinary Research Institute, Westmeadows, Victoria; LR, Leon Rosen, Pacific Biomedical Research Centre, University of Hawaii at Manoa.

6% divergence at the nucleotide level. Cluster A (Fig. 4) contained isolates from all regions represented in this study. This indicates a wide geographic spread of this genetic type, which is inconsistent with the limited dispersal of the suspected vertebrate hosts (macropods and other marsupials). Isolate T48 is linked in this cluster, although it is quite distant from all the other members.

The two remaining clusters (B and C; Fig. 4) consist of three and four isolates, respectively. Cluster B contains isolates from one geographical region (Charleville, Queensland) obtained over a 5-month period. The other cluster contains isolates from New South Wales, Queensland, and the Northern Territory (cluster C; Fig. 3). It should be noted, however, that the Queensland isolate in this cluster (19575) is linked at a lower level than are the other members.

Rearrangement of the order of input of the isolates into the program used to produce the phenograms did not alter the position of isolate 19575, or any other isolate, with respect of the three major clusters within the T48 topotype. Indeed, the cluster structure was quite stable regardless of method used

FIG. 1. Diagrammatic representation of the RR virus genome (RRVNBCG, published sequence of NB5092 [14]) showing the location of the 126 RNase T_1-resistant oligonucleotides equal to or greater than 11 nucleotides in length as determined by computer analysis.

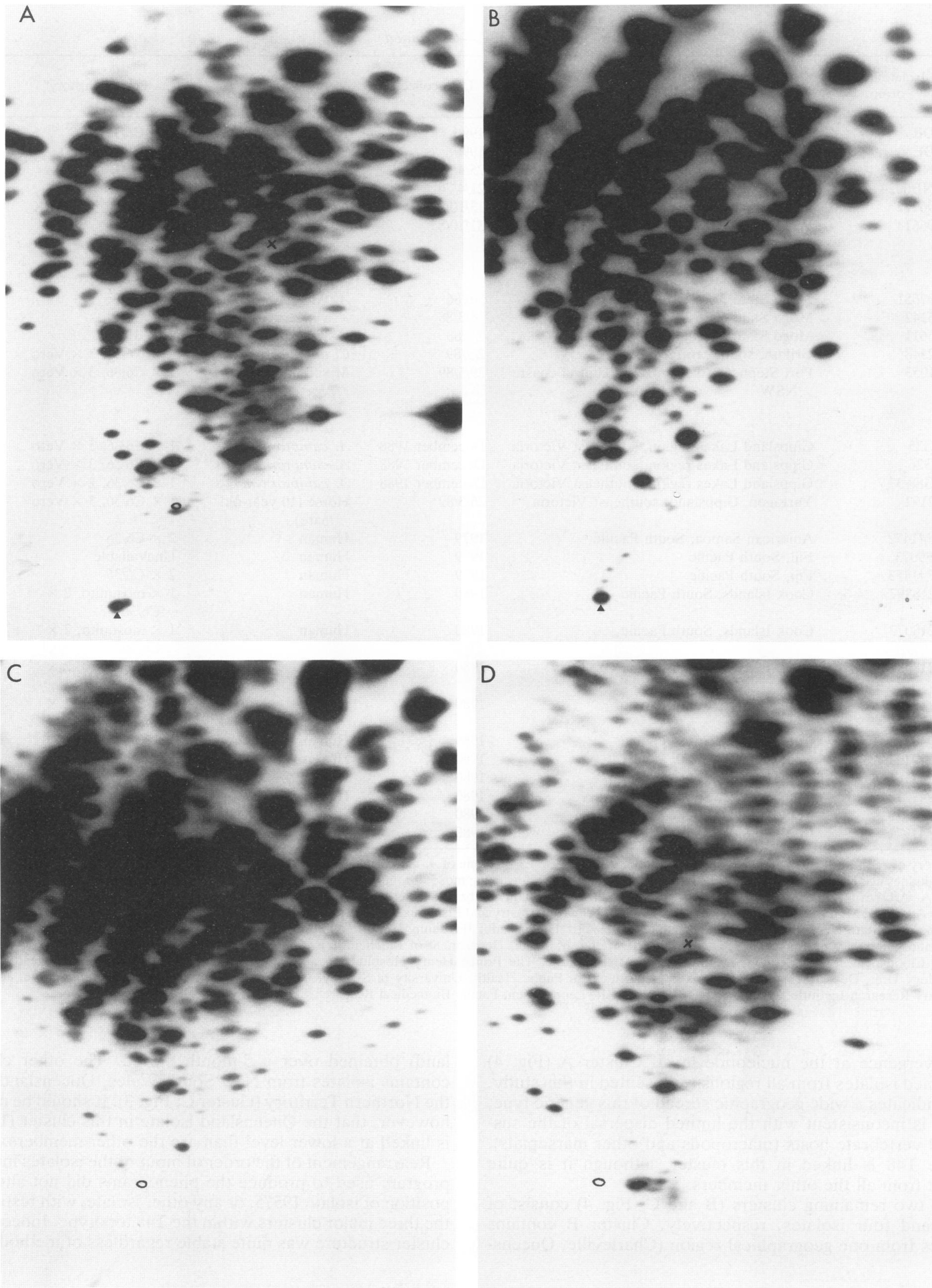

FIG. 2. Autoradiographs of RNase T_1 maps of representative isolates from each of the four topotypes of RR virus found in this study. (A) WK20; (B) T48; (C) K1503; (D) SW 2191. The polyadenylate tail is visible only on the autoradiograph of isolate K1503. Positions of the xylene cyanol FF dye marker (circle) and the bromophenol blue marker (cross) are indicated. The spot that represents a 45-mer in panels A and B is indicated by the symbol ▲.

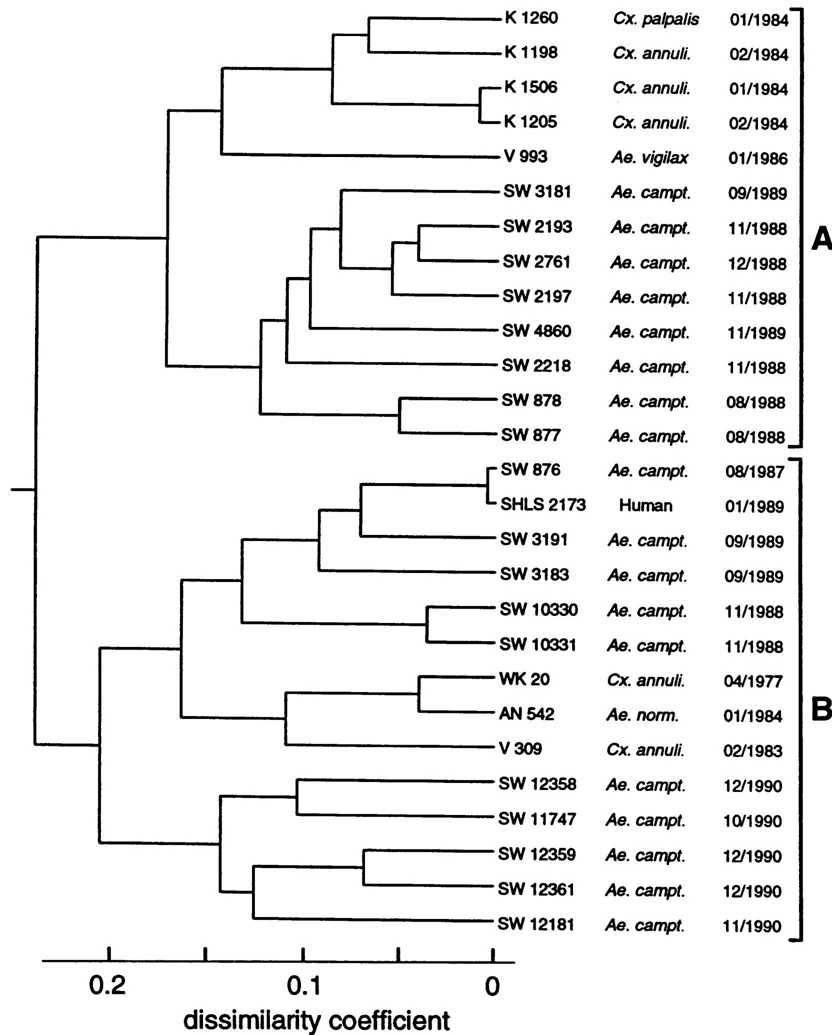

FIG. 3. Phenogram showing the level of dissimilarity of the RNase T_1 maps of the 27 RR virus isolates which were grouped into the WK20 topotype. Host of origin and date of collection of each isolate are also shown. The phenogram was constructed from a distance matrix by using the UPGMA method and the PHYLIP computer package (version 3.4) (16). The two groups of isolates which linked at the lowest level are shown as clusters A and B. The distance between two isolates, or groups of isolates, is read directly from the point of convergence.

(UPGMA, neighbor joining). This indicates that the classification shown here is reasonably stable, as judged from the oligonucleotide maps.

Isolates SW2191 and K1503, each of which is substantially different from all other isolates, both come from Western Australia, the region in which the WK20 topotype was most prevalent. The occurrence of these two strains, one in the Kimberley region (K1503) and one in the southwest (SW2191), is further evidence that cocirculation of substantially different strains of RR virus may occur.

DISCUSSION

Our results show that the genotypes of most RR virus isolates from the southwest and some of the isolates from the Kimberley region in the north of Western Australia differ markedly from the genotypes of those in eastern Australia. The two genotypes predominate in their respective geographical areas over time; however, that was the only detectable association between genotype and region of geographical origin. Thus, most of the isolates examined fit into a pattern that shows two separately circulating major topotypes, one in the east and one in the west of Australia, which meet and to a certain extent overlap in the Northern Territory and the Kimberley region of the north of Western Australia (Fig. 5). In addition, all isolates from the Pacific Islands were grouped in the eastern Australian topotype.

RNase T_1 oligonucleotide maps cannot be reliably compared when sequence divergence at the nucleotide level approaches 8 to 10% (48). Therefore, some isolates from the Kimberley region and from the Northern Territory and all of the isolates from the eastern states (Queensland, New South Wales, and Victoria) and the Pacific Islands (T48 topotype; Fig. 4), which appear to be closely related to each other, can be assumed to have less than 8 to 10% divergence at the nucleotide level. We attempted to compare some isolates from the WK20 topotype with some from the T48 topotype; while apparent matches were observed, several different alignments were possible. This decreased our confidence in the alignment, and therefore we have placed them in differ-

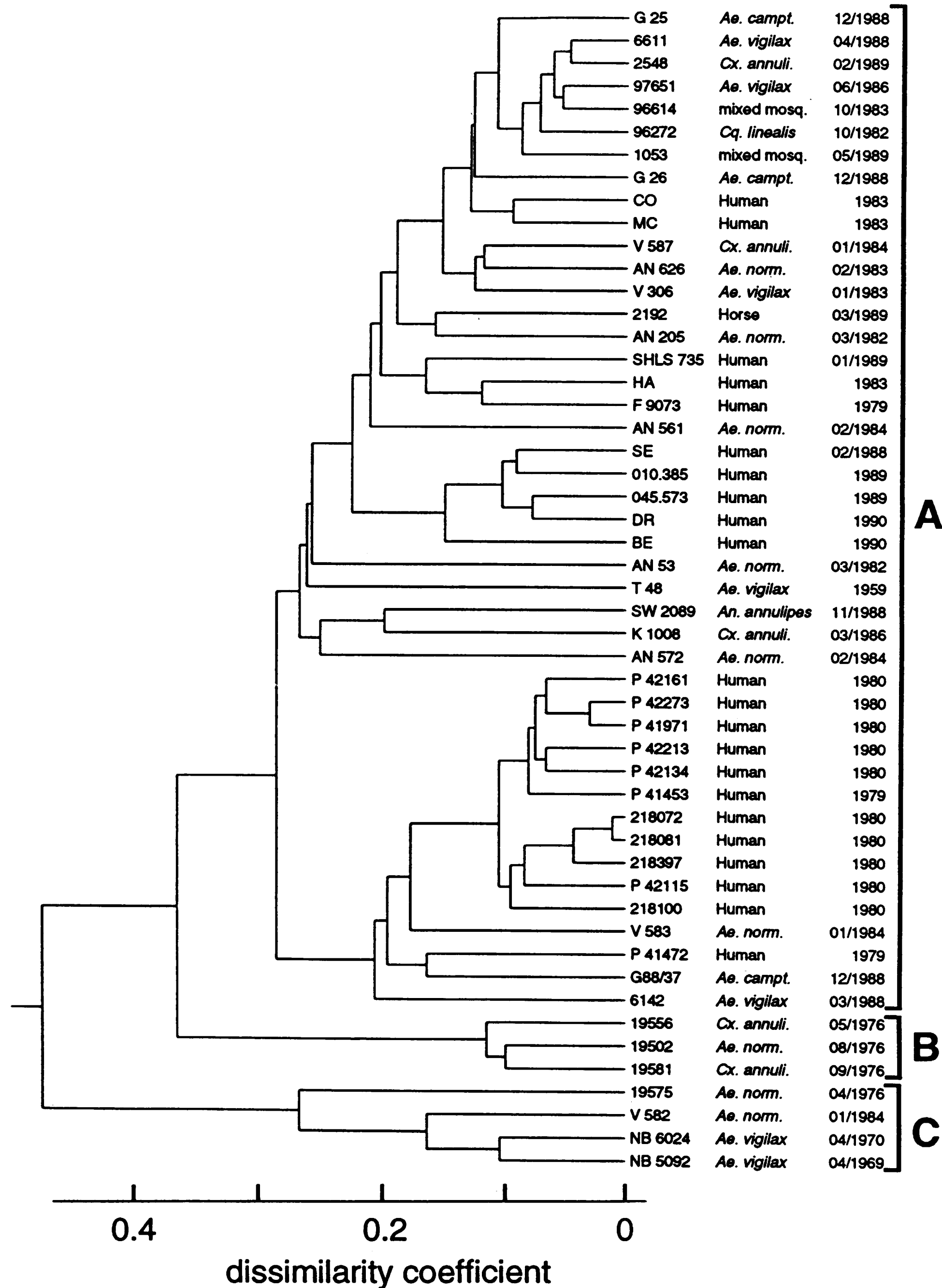

FIG. 4. Phenogram showing the level of dissimilarity of the RNase T_1 maps of the 51 RR virus isolates which were grouped into the T48 topotype. Host of origin and date of collection of each isolate are also shown. Three groups of isolates which linked at the lowest level are shown as clusters A, B, and C. The distance between two isolates, or groups of isolates, is read directly from the point of convergence.

ent topotypes. It should be noted, however, that the different alignments all resulted in a link between the two topotypes at a distance of 0.48. This level equates to approximately 5% nucleotide divergence (± 2 standard deviations range is 3.2 to 7.5%; results not shown). Therefore, the divergence between these two major topotypes is of that order, as measured over the entire genome. Indeed, computer analysis of the RNase T_1-resistant fragments of a published RR virus sequence (14) revealed an even distribution of RNase T_1-resistant fragments longer than 11 nucleotides (Fig. 1).

Two RNase T_1 maps, those of K1503 (Fig. 2C) and SW2191 (Fig. 2D), were unlike those of any other isolate. These strains therefore represent two topotypes that cocirculated with the WK20 topotype. Since we have not found any other isolates with similar RNase T_1 maps, either at different times or in a different geographical region, we cannot determine whether K1503 and SW2191 were incursants from regions that we have not sampled or whether these strains are continuously circulating in the regions where they were found. K1503 was isolated at the same time

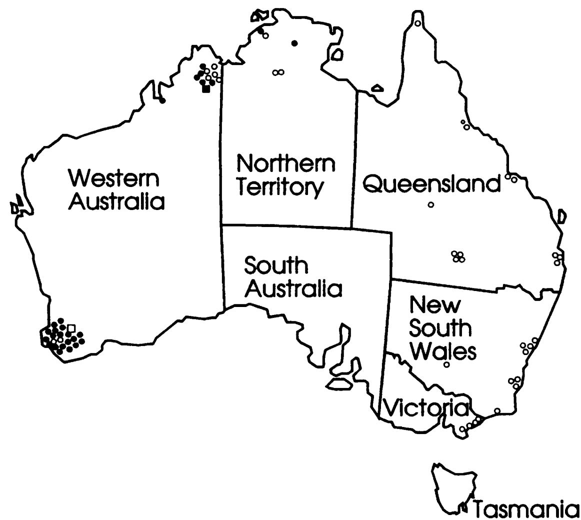

FIG. 5. Map of Australia showing locations of origin of isolates of RR virus from the four different genetic types (topotypes) identified in this study. Isolates belonging to the WK20 topotype are shown as dark circles, and those grouped in the T48 topotype are shown as open circles. The topotype represented by the single isolate SW2191 is shown as an open square, and the topotype represented by the single isolate K1503 is shown as a closed square. The 13 isolates from the Pacific Islands which were all grouped in cluster A of the T48 topotype are not shown.

(in the middle of the wet season) and from the same mosquito vector (*C. annulirostris*) as most other WK20-like isolates. In contrast, SW2191 was isolated in November 1988 at the outset of the largest recorded epidemic of RR virus in the southwest of Western Australia. At this time of the year the level of nonimmune vertebrate hosts in the population is highest and thus conducive to uptake of incursants or amplification of locally circulating viruses. It appears that the SW2191 topotype did not become established, since almost all subsequent isolates from the southwest belonged to the WK20 topotype. At the same time, isolate SW2089, which belongs to the T48 topotype, was obtained during a large surveillance program in which about 110,000 mosquitoes from the southwest were processed (26). It appears that this topotype persisted to some extent, because 2 months later another member of the T48 topotype (SHLS735) was isolated from the serum of a polyarthritis patient from the same region. It is possible, given the fact that birds are not thought to be suitable hosts of RR virus, that this topotype was introduced into the region by a viremic air traveller or domestic livestock carried by air. Indeed, Marshall and Miles (28) postulated that the introduction of RR virus into and among the Pacific Islands has occurred by one or another of such routes.

Previous studies at the genome level on limited numbers of RR virus isolates from eastern Australia and the Pacific Islands (13) found several different genetic types with an estimated sequence divergence of 1.5 to 5%. In general, the clustering within the T48 topotype agrees with these genetic types. The exception is the alignment of isolate 19575, which in our analysis clustered with the Nelson Bay isolates (NB5092 and NB6024). The reason for this difference may be the amount of genome examined by the two different methods (*Hae*III and *Taq*I restriction digests versus RNase T_1 mapping). The two restriction enzymes combined examined about 4% of the genome, whereas our RNase T_1 mapping study (120 nucleotides at a minimum of 11 nucleotides) examines at least 10% of the genome.

Some variation in dominance of different RR virus strains may be taking place in the southwest of Western Australia. No viruses isolated after 1989 in the southwest of Western Australia belong to cluster B (Fig. 3), suggesting that cluster B southwest strains disappeared after 1989. Isolates SW3181 (cluster B) and SW3183 (cluster A) came from two pools of *A. camptorhynchus* caught in the same trap on the same day, suggesting the existence of cocirculating strains. At the time that isolates SW11747, SW12181, SW12358, SW12359, and SW12361 (Fig. 3) were circulating, no other coincident strains were found. Since we do not have data on viruses collected over a long period of time, it is not possible to discern whether this represents evolutionary change of the existing genetic type or whether another genetic type was imported and replaced the originally circulating type. A similar situation may have occurred in the Nelson Bay area, where a strain represented by isolates NB5092 and NB6024 may have been replaced with the strain represented by isolate 1053 (Fig. 4). It is interesting that the genetic type observed during a major epidemic in the southwest of Western Australia was also found in the winter of a year when there was no epidemic (e.g., SW876 and SW877). This finding suggests that establishment of an epidemic need not necessarily be preceded by the introduction or evolution of a new strain of RR virus to an area and that epidemics are driven by environmental conditions that favor the enhanced transmission of the virus between vectors and nonimmune hosts.

The distribution of the two most represented topotypes covers most of the Australian continent, but the demarcation between the two is not clear, and incursants from the T48 topotype have been found in the southwest of Western Australia (SHLS735 and SW2089). Furthermore, the Northern Territory and the northwest of Western Australia harbor isolates from both major topotypes.

It is clear that the ecology of RR virus is different from that of other important mosquito-borne arboviruses in Australia. The flaviviruses MVE (9) and KUN (17) viruses and the alphavirus SIN virus (3) were found to have only minor variants of single dominant topotypes distributed across the Australian continent. Our study shows that the genotype of RR virus is more variable than that of Australian MVE, KUN, and SIN viruses and that some association between genotype and region of geographical origin, albeit eastern Australia versus western Australia, occurs for RR virus. MVE, KUN, and SIN viruses are thought to have waterbirds as their major vertebrate hosts. Migratory waterfowl probably distribute these viruses throughout the continent at regular intervals. On the other hand, RR virus is thought to have marsupials and other terrestrial vertebrates as major hosts, which would not be conducive to rapid transport of the virus from one region to another. However, our results suggest that RR virus ecology is far more complex than that of a virus which simply circulates and evolves in geographically isolated regions. Increasing frequency of air travel by humans and their livestock may result in the distribution of the virus across the continent, or parts of it, quite effectively. If this is the case, then it may now be impossible to accurately determine when and where RR virus strains originated.

The distribution of RR virus isolates into different clusters did not align with source host or vector. This finding is in agreement with the restriction enzyme study of Faragher et

al. (13). Furthermore, biological variants (T48, NB5092, and NB6024) (47) could not be distinguished on the basis of the clusters derived by analysis of the RNase T_1 maps. This is different from the situation described for Venezuelan equine encephalomyelitis (VEE) virus, in which isolates from different geographical regions have different antigenic properties (49) and yield substantially different RNase T_1 maps (40).

The pattern of distribution of RR virus, that is, two topotypes, both of which can be found in tropical and temperate regions, is unlike that of eastern equine encephalomyelitis (EEE) virus (45), VEE virus (40), western equine encephalitis (WEE) virus (41), or Getah virus (29); in these cases, distributions of one topotype do not extend from temperate to tropical regions. Serological, antigenic, and molecular comparison of these other alphaviruses has provided important information about their maintenance and circulation in nature and the involvement of strains in epidemics. Serological analysis of isolates of VEE virus by Young and Johnson (49) showed that several different antigenic subtypes and varieties of this virus exist and that place of origin and, to a lesser extent, time were important determinants of antigenic variation. This finding was consistent with field studies which showed that small forest rodents and *Culex* (*Melanoconion*) mosquitoes were probably the maintenance hosts and vectors of enzootic strains of the virus (19). Molecular comparisons of isolates of VEE virus by using RNase T_1 mapping (5, 40) supported the antigenic grouping of Young and Johnson. As discussed above, distinct differences were found in the RNase T_1 maps of isolates from the different antigenic subtypes and varieties but not between isolates from within each subtype or variety.

In contrast, WEE virus strains from widely separated regions and with distinct isolation histories exhibit greater than 90% RNase T_1 map homology (41). This uniformity suggests that WEE virus may be continuously exchanged by intercontinental transport in birds, thought to be the principal vertebrate hosts of the virus. Alternatively, stringent ecological selection factors may be preventing the evolution of more divergent genotypes (32).

In North America, EEE virus, like WEE virus, exhibits extreme genetic homogeneity (33). The rate of evolution of EEE virus in North America has also been shown to be extremely low (46). This observation was attributed by Weaver et al. (45) to specific adaptation to a principal mosquito vector (*Culiseta melanura*), slower rates of virus replication, and mobility of the main vertebrate hosts, passerine birds. However, in tropical America where some species of rodents and other small mammals are important hosts, EEE virus isolates show considerable genetic divergence, more like that observed for VEE virus (45). Such divergence is partly attributed to virus adaptation to small, disconnected environments and adaptation to transmission by several different mosquito species.

All isolates of the alphavirus Getah virus, obtained from the same locality in Japan in the same year, had different RNase T_1 maps (29). Birds, the only vertebrate hosts known to be capable of introducing new variants of the virus on such a frequent basis, are not thought to be reservoirs (25). This suggests that Getah virus, unlike RR virus (4), undergoes frequent mutation during transmission cycles between relatively sedentary hosts (horses and pigs) and mosquitoes.

In view of the apparent differences in the ecology of RR virus and these other alphaviruses and the complex nature of the observed distribution of RR virus isolates, further investigations into transmission cycles of RR virus need to be carried out. In particular, the role of humans, birds, and domestic livestock in amplification and movement of the virus should be examined.

Although no evidence of recombination in alphaviruses was observed in temperature-sensitive mutants of either SIN or Semliki Forest virus (31), sequencing data obtained by Hahn et al. (20) suggest that WEE virus may have arisen (naturally) by recombination between an EEE-like virus and a SIN-like virus. The question of recombination between different strains of RR virus or between RR virus and other alphaviruses cannot be addressed using our data because RNase T_1 oligonucleotide analysis does not permit identification of the location of oligonucleotides within the genome. This, in turn, means that it is not possible to discern whether the observed changes are due to recombination or evolution (drift). In view of extremely high isolation rates of RR and other alphaviruses in some studies with field-caught mosquitoes from the same location (25a), this question needs to be answered. To do this, observed differences in genotype between RR virus isolates must be further defined. Cycle sequencing of a large number of RR virus isolates is currently in progress.

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