

Yellow Fever Virus Maintenance in Trinidad and Its Dispersal throughout the Americas[†]

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Received 18 March 2010/Accepted 6 July 2010

Trinidad, like many other American regions, experiences repeated epizootics of yellow fever virus (YFV). However, it is unclear whether these result from *in situ* evolution (enzootic maintenance) or regular reintroduction of YFV from the South American mainland. To discriminate between these hypotheses, we carried out a Bayesian phylogeographic analysis of over 100 prM/E gene sequences sampled from 8 South American countries. These included newly sequenced isolates from the recent 2008–2009 Trinidad epizootic and isolates derived from mainland countries within the last decade. The results indicate that the most recent common ancestor of the 2008–2009 epizootic existed in Trinidad 4.2 years prior to 2009 (95% highest probability density [HPD], 0.5 to 9.0 years). Our data also suggest a Trinidad origin for the progenitor of the 1995 Trinidad epizootic and support *in situ* evolution of YFV between the 1979 and 1988–1989 Trinidad epizootics. Using the same phylogeographic approach, we also inferred the historical spread of YFV in the Americas. The results suggest a Brazilian origin for YFV in the Americas and an overall dispersal rate of 182 km/year (95% HPD, 52 to 462 km/year), with Brazil as the major source population for surrounding countries. There is also strong statistical support for epidemiological links between four Brazilian regions and other countries. In contrast, while there were well-supported epidemiological links within Peru, the only statistically supported external link was a relatively weak link with neighboring Bolivia. Lastly, we performed a complete analysis of the genome of a newly sequenced Trinidad 2009 isolate, the first complete genome for a genotype I YFV isolate.

Yellow fever virus (YFV) is historically one of the most important arboviral pathogens. This single-stranded positive-sense RNA virus (family *Flaviviridae*, genus *Flavivirus*) may be maintained either by a sylvatic transmission cycle involving nonhuman primates and forest canopy-dwelling mosquitoes (28) or by an urban human-mosquito-human cycle mediated by the urban-dwelling, anthropophilic *Aedes aegypti* mosquito (36). In the Americas, where the virus was introduced from Africa approximately 300 to 400 years ago (4), the primary sylvatic vectors are mosquitoes of the *Haemagogus* and *Sabethes* genera.

Despite the existence of a highly effective vaccine, available since 1937, and concerted efforts to eradicate *A. aegypti*, the failure to sustain vaccination and mosquito control programs has resulted in YFV continuing to be an important public

health concern, causing an estimated 200,000 cases and 30,000 deaths each year (44). The patterns of viral activity in Africa and South America are, however, markedly different. On the African continent, there are regular epidemics, while in South America, the last major urban epidemic vectored by *A. aegypti* (i.e., with confirmed human-mosquito-human transmission) occurred in Brazil in 1928 (40). Since then, there have been only a small number of sporadic cases reported, in Brazil (3 cases in 1942) (18), Trinidad (15 cases in 1954) (28), and Bolivia (6 cases in 1999) (45). There is, however, regular epizootic activity in neotropical forested areas, with occasional spillover into human populations in surrounding rural areas. These epizootics appear to be cyclical in nature, occurring approximately every 5 to 10 years in a given geographic area. The most recent began in October 2008 and affected Argentina, Brazil, Colombia, Venezuela, and Trinidad (31). The onset of these epizootics is typically signaled by findings of dead monkeys in forested areas, as most New World primates are highly susceptible to infection and many die (especially *Alouatta* sp. howler monkeys). It has been suggested that the observed periodicity between epizootics reflects the time necessary for renewal of the susceptible monkey populations (7, 48). However, it is still unclear how YFV survives during in-

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† Supplemental material for this article may be found at <http://jvi.asm.org/>.

[‡] Published ahead of print on 14 July 2010.

TABLE 1. Viruses sequenced in this study and associated information

Isolate	Source	Location	Yr of isolation	Passage history ^d	GenBank accession no.
06-15094-99 ^a	Human	Peru	1999	C6/36#2	HM582846
BeAr527785 ^a	<i>Sabethes</i> sp.	Brazil	1994	SM1, C6/36#1	HM582845
BeAr631464 ^a	<i>Sabethes chloropterus</i>	Bolivia	2001	SM1, C6/36#1	HM582848
BeH613582 ^a	Human	Brazil	1999	SM1, C6/36#1	HM582843
BeH622205 ^a	Human	Brazil	2000	SM1, C6/36#1	HM582849
FMD1240 ^a	Human	Peru	2007	C6/36#2	HM582844
FSC0737 ^{a,b}	Human	Peru	2005	Vero1, C6/36#1	HM582850
FVB0196 ^a	Human	Bolivia	2006	C6/36#2	HM582847
TVP11640 ^a	<i>Alouatta seniculus</i>	Trinidad	2009	C6/36#1	HM582842
TVP11646 ^a	<i>Alouatta seniculus</i>	Trinidad	2009	Vero1	HM582839
TVP11649 ^a	<i>Alouatta seniculus</i>	Trinidad	2009	Vero1	HM582840
TVP11687 ^a	<i>Alouatta seniculus</i>	Trinidad	2009	Unpassaged	HM582841
TVP11767 ^{a,c}	<i>Alouatta seniculus</i>	Trinidad	2009	C6/36#1, Vero1	HM582851

^a Only prM/E genes were sequenced.

^b Vaccine strain based on ML phylogeny.

^c prM/E genes and complete genome were sequenced.

^d SM, suckling mice; C6/36, *Aedes albopictus* cells; Vero, African green monkey kidney cells.

terepizootic periods. Four possibilities, previously noted by Bryant et al. (3), are as follows.

(i) Wandering epizootics are scenarios in which “epizootics in nonhuman primate species move continuously throughout the Amazon region. . .from one susceptible population to another” (3). The occurrence of wandering epizootics was the most widely accepted hypothesis, but more recent work suggests enzootic maintenance of YFV in both Peru (3) and Brazil (49).

(ii) Persistent infection in monkeys has been documented for some primate species in the laboratory, but persistent viremia levels appear to be insufficient to infect vectors (33, 54).

(iii) There is evidence of vertical transmission in mosquitoes in Senegal, where YFV has been isolated from male *Aedes furcifer-taylori* (8) and *A. aegypti* (12, 20) mosquitoes. Transovarial transmission has also been reported for *Haemagogus equinus* (17) and *Aedes* sp. (2) mosquitoes. Additionally, there is evidence of vertical transmission in *Haemagogus janthinomys*, the principal vector in South America, based on virus isolation from nulliparous females (29). More recently, Sall et al. (38) suggested that the low evolutionary rate observed for YFV compared to that for dengue virus may be a consequence of vertical transmission.

(iv) An alternative unknown transmission cycle may exist. There is recent serological evidence that agoutis (*Dasyprocta leporina*) are frequently infected (10), but it is still not clear whether these are amplifying or dead-end hosts.

None of the aforementioned scenarios are mutually exclusive, and the relative contribution of each may vary from place to place. More-effective monitoring and control of YFV might therefore hinge on the development of site-specific programs that respond appropriately to local scenarios. The question of whether YFV is periodically imported from other regions or maintained locally between outbreaks is particularly pertinent for the island of Trinidad. The recent 2008–2009 epizootic in Trinidad was heralded by reports of dead howler monkeys in the southeastern regions of the island in October 2008, and within 3 months, dead monkeys were reported in the far north-eastern region of the island. Prior to this, epizootic YFV activity occurred in 1979, 1988–1989, and 1995. In order to de-

termine whether there is *in situ* evolution (enzootic maintenance) of YFV between epizootics in Trinidad or if the virus is regularly reintroduced from the mainland, we carried out a phylogeographic analysis of YFV in the Americas based on sequence data from 8 countries, spanning 55 years. Our data set of 104 sequences from the Americas included newly derived sequences from five YFV isolates from the 2008–2009 epizootic in Trinidad and 8 isolates from the mainland sampled between 1994 and 2009.

We employed a powerful Bayesian phylogeographic approach (22) to reconstruct the transmission of the virus through space and time and to infer the geographic locations of ancestral viral lineages. Additionally, we estimated evolutionary rates and dates of divergence for YFV in the Americas as well as for individual clades, including that representing the 2008–2009 epizootic in Trinidad. We also describe the complete genomic sequence of one isolate from the recent Trinidad epizootic.

MATERIALS AND METHODS

Virus isolation and propagation. Virus isolates were derived from liver tissues sampled from dead *Alouatta* sp. monkeys ($n = 11$) obtained by the Trinidad and Tobago Public Health Laboratories from the Bush Bush Forest, Biche, Guayaguayare, Chaguaramas, Plum Mitán, and Maracas areas during the 2008–2009 epizootic in Trinidad. Samples were stored at -70°C and then shipped to the University of Texas Medical Branch (UTMB) for virus isolation via cell culture. The samples were homogenized using a TissueLyser (Qiagen, Valencia, CA) and then centrifuged at 10,000 rpm for 5 min, and the supernatants were collected. For each sample, 200 μl of supernatant was inoculated onto C6/36 mosquito cells and incubated at 28°C for 7 days. After 1 week, a portion of the C6/36 cells was examined by an indirect fluorescent-antibody test (IFAT) as described previously (43). In addition to the above-mentioned isolates, eight additional strains, isolated from Peru ($n = 3$), Brazil ($n = 4$), and Bolivia ($n = 1$) between 1994 and 2009, were selected from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) collection at UTMB (Table 1).

Viral RNA extraction, amplification, and sequencing of prM/E gene region. RNAs were extracted from 140 μl of cell culture supernatant by use of Qiagen viral RNA extraction kits according to the manufacturer’s instructions (Qiagen, Valencia, CA). Reverse transcription-PCR (RT-PCR) was then used to amplify a prM/E gene fragment consisting of 334 nucleotides (nt) of the prM gene and 336 nt of the E gene. This region has been sequenced extensively in the past, thus facilitating a more detailed phylogenetic analysis (3, 4, 46). Amplification was performed using a Titan one-tube RT-PCR kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions, except for

TABLE 2. Primer pairs used for RT-PCR amplification and sequencing of the prM/E gene region and the complete genome

Sense primer	Position	Melting temp (°C)	Sequence	Antisense primer	Position	Melting temp (°C)	Sequence	Product size (bp)
B5	1	61.3	AGT AAA TCC TGT GTG CTA ATT GAG G	YF7	1312	53.7	AAT GCT TCC TTT CCC AAA T	1,312
AJ7up	1223	64.6	GAA GAG AAC GAA GGG GAC AAT GC	YF100	2554	60.6	GTA TGA GTA CTT GTT CAG CCA GTC	1,331
sNS1A	2394	65.4	CCA TGA GCA TGA TCT TGG TAG G	YF3600a	3600	64.3	GCA CCA TGC CTC CGA C	1,206
sNS3C	5174	64.5	GCT GGG AAG ACA AGA CGT TTC C	YF6400a	6400	62.3	GAC CAG CAT CTC CAG CAG	1,226
NS4A1	6338	69.4	GGA GGA GCA AAG AAG CCT CTG C	aNS5a	8352	64.5	AGG CGG GAT GTT TGG TTC ACA G	2,014
FU2	9233	83.1	GCT GAT GAC ACC GCC GGC TGG GAC AC	aNS5i	10862	59.9	AGT GGT TTT GTG TTT TTC ATC CAA AG	1,629
NS4A1	6338	69.4	GGA GGA GCA AAG AAG CCT CTG C	YF9334a	9334	68.9	GTA AGT CAT TTC CAT TAC AGC CAG CG	2,996
YF3400s	3420	53.3	GGC TTT CAC GGG AGT	YF5300a	5300	72.5	TCA AGG TAG CAT GAC ACA TAG CGT CAA TG	1,880
NS1c	2984	64.6	GAC TGC GAT GGA TCT ATC TTG GGT	YF5300	5300	72.5	TCA AGG TAG CAT GAC ACA TAG CGT CAA TG	2,316
CAG ^a	625	50.3	CTG TCC CAA TCT CAG TCC	YF7 ^a	1312	53.7	AAT GCT TCC TTT CCC AAA T	687
EMF	9988	50	TGG ATG ACS ACK GAR GAY AT	VD8	10700	53.83	GGG TCT CCT CTA ACC TCT AG	712

^a Used for amplification and sequencing of the prM/E gene region. All other primers were used for amplification and sequencing of the complete genome. Primer positions are based on the ASIBI strain.

the following adjustments: we used 3 mM MgCl₂, 2 µl of enzyme mix, and 0.5 µl of RNasin. Thermocycling conditions for the prM/E gene region were adapted from the work of Wang et al. (51) and were as follows: 45°C for 45 min, 95°C for 2 min, and 36 cycles of 95°C for 10 s, 55°C for 30 s, and 68°C for 1 min, with a final extension at 68°C for 2 min.

Amplicons were visualized in a 1.5% agarose gel stained with ethidium bromide and were excised and purified using a QiaQuick gel extraction kit (Qiagen, Valencia, CA) before being subjected to direct sequencing using a BigDye Terminator v3.1 cycle sequencing kit (Roche) and an Applied Biosystems 3100 genetic analyzer. Sequence cycling conditions were as follows: 96°C for 2 min and 35 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Primers used for amplification and sequencing are shown in Table 2.

Complete genome sequencing of isolate TVP11767. Isolate TVP11767 was propagated by inoculation onto Vero cells. Once cytopathic effects (CPE) were detected, viral RNA was extracted using a Qiagen viral RNA extraction kit (Qiagen, Valencia, CA). The complete genome was then amplified as overlapping fragments and sequenced with primers designed relative to the ASIBI strain. PCR conditions were as follows: 45°C for 45 min, 95°C for 2 min, and 36 cycles of 95°C for 10 s, 50°C for 30 s, and 68°C for 1 min, with a final extension at 68°C for 2 min. The PCR amplicons were visualized, gel purified, and sequenced as described above.

Data sets. Using the Se-AL program (<http://tree.bio.ed.ac.uk>), the newly derived prM/E gene sequences from Trinidad, Brazil, Bolivia, and Peru were manually aligned with YFV sequences from the Americas, obtained from GenBank and trimmed to a common length of 654 nt. Previously identified vaccine strains and strains associated with YFV vaccination adverse reactions were excluded. Of all the sequences generated during this study, only the Peru 2005 sequence grouped with vaccine strains in a preliminary phylogenetic analysis (not shown), and this strain was therefore removed from all but the BaTS analysis. A Brazil 1935 sequence was also removed because it had undergone extensive mouse brain passages and, furthermore, its reported sampling time appeared to be strongly at odds with its root-to-tip genetic distance, as revealed using the linear regression method implemented in Path-O-Gen (<http://tree.bio.ed.ac.uk>) (data not shown). The final data set consisted of 104 taxa (see Table S1 in the supplemental material). All sequences were confirmed as nonrecombinant by using the various methods for recombination detection implemented in GARD (35) and RDP3 (23, 24). A map illustrating the geographic distribution of the individual sequences included in this study is shown in Fig. S1 in the supplemental material.

Using Se-AL, the complete genome sequence of TVP11767 was manually aligned with 17 complete YFV genome sequences available in GenBank. The

latter included 8 wild-type strains and 9 vaccine strains or strains associated with a YFV vaccination adverse reaction. Noncoding regions were removed so that only the complete polypeptide open reading frame (10,248 nt) was used for analysis.

Quantifying the extent of geographic structure in the YFV phylogeny. The extent of geographic structure in a YFV phylogeny of 106 prM/E gene sequences was assessed using the program BaTS (32) with Bayesian Markov chain Monte Carlo (MCMC) trees generated using BEAST software. This approach accounts for uncertainty in the underlying phylogeny, as inferences are made over all plausible trees. BaTS quantifies the level of association between the phylogenetic position and country of origin of each strain by using two statistics: the association index (AI) (53) and the parsimony score (PS) (19). The approach provides 95% confidence intervals for each statistic and accounts for phylogenetic uncertainty by integrating across all plausible trees. Overall geographic structure was investigated by assigning a character state to each sequence that represents its country of origin (8 states). In addition, the degree of geographic clustering for each country was investigated by assigning a binary character state to each sequence that denotes whether it was sampled from the country of interest or not.

Phylogeographic reconstruction of YFV spread through time and space. The BEAST (v1.5.2) software package (15) includes a discretized spatial diffusion model, recently described by Lemey et al. (22), which can quantify the rate at which viral lineages move among sampled locations. The BEAST approach enables one to jointly estimate nucleotide substitution rates, divergence times, spatial diffusion, and demographic history from sampled YFV sequences, while concurrently taking into account phylogenetic uncertainty arising from both the sequence data and the spatial diffusion process. Analyses were carried out using a general time reversible model with a discretized, gamma-distributed across-site rate variation (GTR+Γ₄) substitution model, an uncorrelated lognormal molecular clock model (13), and a Bayesian skyline plot (BSP) model (16). The uncorrelated lognormal molecular clock was selected over a strict clock based on the results of the linear regression method implemented in Path-O-Gen (<http://tree.bio.ed.ac.uk>) and on data from the work of Sall et al. (38) indicating deviation from a strict clock. To ensure statistical efficiency, we also employed a Bayesian stochastic search variable selection (BSSVS) procedure (22). Briefly, BSSVS imposes a prior distribution on the spatial model that favors a limited number of nonzero movement rates among pairs of locations, thus reducing the number of estimated parameters and identifying only those rates that are required to explain the observed data (22). As proposed by Lemey et al. (22), we used a truncated Poisson prior with a mean of log 2 and an offset of $K-1$, where K represents the number of discretized location states. We explored equal-rate

priors and distance-informed rate priors for “among-country” and “among-region” movement matrices (see below). To measure the number of independent virus introductions into particular locations and to estimate overall dispersal rates, we employed Markov chain induced counting (jump) process theory (25, 26) and robust counting methods to count labeled location state exchanges along a posterior distribution of phylogenies (30). These tools provide efficient analytical solutions for counting the expected number of unobserved transitions along branches in these trees. Robust counting can be considered a semiparametric procedure that further provides protection against model misspecification inherent in describing spatial diffusion through a discretized Markov chain process. By labeling location transitions with the actual geographic distances involved, we obtained a robust estimate of the expected dispersal distances and rates across the tree.

Inferences were obtained using a Bayesian MCMC approach as previously described (14, 15). MCMC analyses were run for 30 to 50 million generations, with a 10% burn-in period (which was more than adequate to achieve stationarity), and data were sampled every 5,000 states. The program Tracer, version 1.4 (<http://tree.bio.ed.ac.uk/software/tracer/>), was used to monitor for stationarity and efficient mixing, as diagnosed using effective sample size (ESS) statistics. Tracer was also employed to perform model comparisons using Bayes factors (BF), which are based on harmonic mean estimates of the model marginal likelihood (41). The program TreeAnnotator, version 1.5.2 (<http://beast.bio.ed.ac.uk>), was used to summarize the posterior tree distribution, and FigTree, version 1.1.2 (<http://beast.bio.ed.ac.uk>), was used to visualize the annotated maximum clade credibility (MCC) tree.

The phylogeographic analyses required that each YFV sequence be assigned a specific “character state” based on its geographic origin. In two separate analyses (among countries and among regions), we considered movement among countries (8 character states, i.e., Bolivia, Brazil, Colombia, Ecuador, Panama, Peru, Trinidad, and Venezuela) and among regions assigned on the basis of geographic clustering of the available isolates (18 character states, i.e., Bolivia; Amapa, Brazil; Maranhao, Brazil; North Central Amazonian Brazil; Para, Brazil; Rondonia, Brazil; East Sub-Amazonian Brazil; West Sub-Amazonian Brazil; Colombia; Ecuador; Panama; Central Peru; North Peru; South Peru A; South Peru B; Trinidad; North Venezuela; and South Venezuela). The character states assigned to each taxon in the data sets and the coordinates (latitudes and longitudes) for the respective countries/regions are summarized in Table S1 in the supplemental material. Where two discrete locations were grouped together, the longitude and latitude used were those of the midpoint of the line connecting them. Where more than two locations were grouped, the latitude and longitude of the centroid of the polygon defined by them were used.

Phylogenetic analysis of complete YFV polyprotein open reading frames. Sequence comparisons to determine the degree of nucleotide and amino acid divergence for the complete polyprotein open reading frame and its individual genes (partitioned based on the 17D reference sequence) were performed using MegAlign from the DNASTar package (DNASTar, Inc., Madison, WI) and a data set of 18 complete polyprotein open reading frames. Table S2 in the supplemental material shows the accession numbers for the strains included in the analysis. PhyML (21) was used to estimate a maximum likelihood (ML) phylogeny under the GTR+I₄ model, which was identified as the best-fit model of nucleotide substitution by FindModel (42). Bootstrapping was performed to assess the robustness of tree topologies, using 1,000 replicate neighbor-joining (NJ) trees under the ML substitution model.

Nucleotide sequence accession numbers. Nucleotide sequences derived for the prM/E gene region and the complete genome sequence of isolate TVP11767 were submitted to GenBank under the accession numbers shown in Table 1.

RESULTS

Quantifying the extent of geographic structure in the YFV prM/E phylogeny. Both the AI and PS statistics indicated that there was very strong geographic clustering of YFV strains by country of origin ($P < 0.05$) (Table 3). When the extent of phylogenetic clustering of individual countries was tested, population subdivision was significant for all countries except for Colombia, Ecuador, and Bolivia, for which there was evidence of significant gene flow to and from other regions of the Americas ($P > 0.05$). This suggests that in all but these three cases, observed YFV genetic diversity is shaped primarily by *in situ* evolution rather than extensive migration. However, the num-

bers of sequences from Colombia, Ecuador, and Bolivia were very small, which may bias the results.

Phylogeographic reconstruction of YFV spread through time and space. Having established the existence of statistically significant geographic structure, as demonstrated by the above-mentioned BaTS analysis, we applied a Bayesian phylogeographic approach in order to investigate how this structure was established. Analyses were performed under two models: (i) an equal rates model that assumes the same rate of virus movement between all pairs of countries and (ii) a distance-informed model that expects the intensity of virus movement between locations to be inversely proportional to the distance between those locations (22). BF comparison of the model marginal likelihoods indicated marginal support for the distance-informed model over the equal rates model (ln BF = 1.6). Since this support is far from overwhelming, we opted for the less complex model and report the remaining results for the analysis using the equal-rate priors. The mean nucleotide substitution rate was estimated to be 3.7×10^{-4} substitution per site per year (95% highest probability density [HPD] = 2.6×10^{-4} to 5.1×10^{-4} substitution per site per year), and the mean estimate for the date for the most recent common ancestor (MRCA) was 1822 (95% HPD, 1701 to 1911).

Figure 1 illustrates the Bayesian MCC tree for YFV in the Americas, including the posterior probabilities (statistical support) for individual clades. The color of each lineage and internal node represents its most probable geographic locality. The previously described South American genotypes I and II (4–6, 50) are distinct in the phylogeny, and the spatial reconstruction suggests that YFV in the Americas most likely originated in Brazil. Genotypes I and II are dominated by Brazilian and Peruvian populations, respectively, but the higher probability of a Brazilian origin (49%) than a Peruvian one (20%) may be explained at least partly by the presence of a basal Brazilian strain in the genotype II cluster (see histogram insert in Fig. 1 for root state posterior probabilities). The 18-location “among-region” analysis also suggests Brazil as the origin, as the sum of the posterior probabilities for all Brazilian locations was 52%, compared to 17% for Peruvian locations (data not shown). The Trinidad 2009 sequences were placed in genotype I, along with all but one of the earlier Trinidad sequences. This exception was a 1979 Trinidad sequence, which grouped most closely, as previously reported, with Peruvian sequences from 1981 (4, 9, 52). Within genotype I, the Trinidad sequences all clustered within the same major clade (posterior probability = 0.75), which also contained Brazilian and Venezuelan sequences.

Dates of divergence for Trinidadian clades are shown in Table 4. The MRCA of the Trinidad 2009 isolates was estimated to have arisen in 2004 (95% HPD, 2000 to 2008), and the mean substitution rate for the clade containing them was estimated to be 2.36×10^{-4} substitution/site/year (95% HPD, 0.41×10^{-4} to 5.0×10^{-4} substitution/site/year). The Trinidad 2009 strains share an ancestor with a basal 1995 Trinidad sequence (posterior probability, >99.9). A similar relationship was seen between the sequences from the 1989 and 1979 Trinidad epizootics. These results provide strong support for the 2008–2009 and 1988–1989 YFV epizootics having evolved within Trinidad from common ancestors of the 1995 and 1979 epizootics, respectively (the probabilities that the ancestral

TABLE 3. Extent of geographic clustering, as indicated by AI and PS statistics

Statistic	Observed value			Null value			<i>P</i> value ^a
	Mean	Lower 95% CI	Upper 95% CI	Mean	Lower 95% CI	Upper 95% CI	
Among countries (8 states)							
AI	3.29	2.66	3.92	8.47	7.46	9.36	0.00
PS	25.71	24.00	27.00	54.62	51.28	57.87	0.00
Peru (<i>n</i> = 30) vs others							
AI	1.51	0.96	2.05	4.50	3.58	5.48	0.00
PS	10.68	9.00	12.00	26.47	23.99	28.56	0.00
Brazil (<i>n</i> = 44) vs others							
AI	2.21	1.80	2.60	5.45	4.35	6.49	0.00
PS	15.44	14.00	17.00	34.12	30.18	37.56	0.00
Colombia (<i>n</i> = 3) vs others							
AI	0.62	0.32	0.87	0.59	0.27	0.89	0.55
PS	2.84	2.00	3.00	2.98	3.00	3.00	1.00
Venezuela (<i>n</i> = 4) vs others							
AI	0.47	0.43	0.54	0.82	0.51	1.15	0.03
PS	3.01	3.00	3.00	3.98	3.87	4.00	0.01
Trinidad (<i>n</i> = 13) vs mainland							
AI	0.18	0.01	0.51	2.43	1.76	3.01	0.00
PS	5.68	5.00	6.00	12.53	11.48	13.00	0.00
Panama (<i>n</i> = 3) vs others							
AI	0.06	0.01	0.26	0.60	0.30	0.90	0.00
PS	2.00	2.00	2.00	2.99	2.98	3.00	0.00
Ecuador (<i>n</i> = 3) vs others							
AI	0.83	0.58	0.97	0.61	0.29	0.92	0.91
PS	3.00	3.00	3.00	2.98	2.97	3.00	1.00
Bolivia (<i>n</i> = 6) vs others							
AI	1.18	0.74	1.61	1.57	1.10	2.08	0.10
PS	7.32	6.00	8.00	7.89	7.00	8.00	0.04

^a Values in bold indicate statistical significance.

nodes were located in Trinidad were 94% and 97%, respectively). There is, however, some evidence of movement from Brazil to Trinidad on longer time scales, as there is an 81% probability that the common ancestor of the 1999–2001 Brazilian clade and the 1995–2009 Trinidad clades was located in Brazil, compared to a probability of only 14% for a Trinidad origin. Similar (albeit weaker) evidence of YFV movement from Brazil to Trinidad is suggested by the posterior probabilities for the node subtending the 1979 and 1988–1989 Trinidad isolates within genotype I and for the 1954 isolate, which were 75% and 63%, respectively, in favor of a Brazilian origin. Movement of YFV into Trinidad from the mainland is also suggested by the location of the 1979 Trinidad isolate within genotype II, which has a 95% probability of originating from Peru. As an additional estimate, we performed Markov chain induced robust counting of independent YFV introductions into Trinidad (Fig. 2), which indeed confirmed three separate introductions from Brazil to Trinidad, generating the 1954, 1979–1989, and 1995–2009 clades, plus one introduction from Peru.

Temporal dynamics of the spatial diffusion of YFV and identification of epidemiologically linked regions. The histories of dispersal of YFV among countries and among regions,

reconstructed using the equal rates model, are shown in Fig. 3. Interactive virtual globe projections demonstrating the diffusion dynamics through time are available online at <http://www.phylogeography.org>. Figures 4a and b show only those linkages that are statistically well supported (i.e., nonzero rates supported by a BF of >3). Using the “among-region” analysis, which incorporates more geographic detail (18 location states), the overall rate of spatial dispersal was estimated to be 182 km/year (95% HPD, 52 to 462 km/year). Since this estimate may be sensitive to how locations are assigned, the analysis was repeated using the most specific locations available for each YFV isolate (28 location states) (data not shown). A very similar estimate of 187 km/year (95% HPD, 89 to 344 km/year) was obtained, indicating that the analysis based on 18 locations had sufficient geographic detail to converge on an accurate estimate.

For the “among-country” analysis, the earliest inferred migration event was from Brazil to Peru between 1822 and 1921. The virus then spread northward from Brazil into Venezuela, Trinidad, and Ecuador and into Panama via Venezuela. Of these links, there is statistical support for all but the links with Ecuador and Colombia. The data suggest that virus subsequently radiated out of Peru—north to Ecuador, northeast to

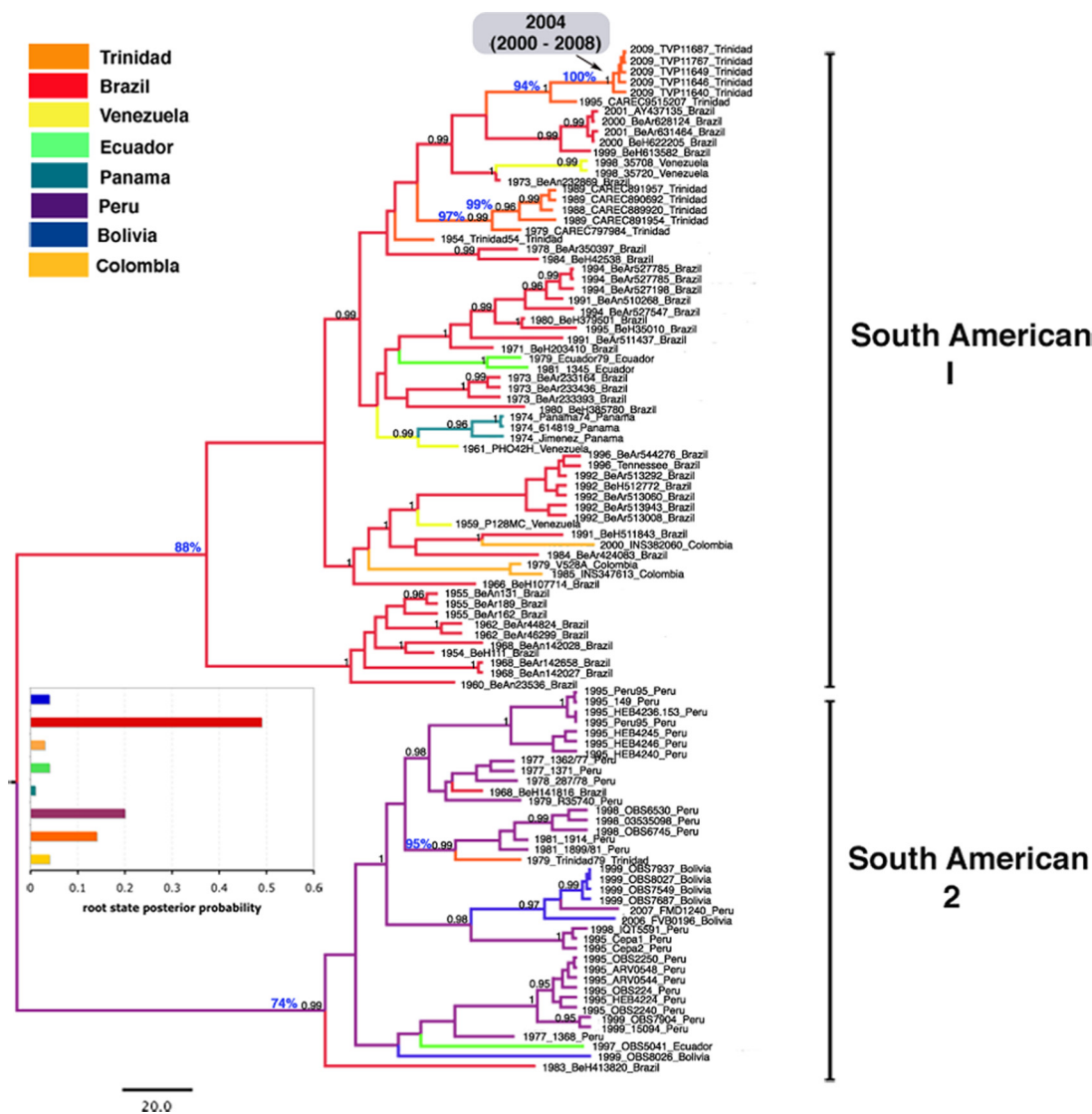


FIG. 1. Bayesian MCC tree for YFV in the Americas, based on prM/E gene fragment of 654 nt. Taxon labels include year of isolation, strain designation, and country of isolation. Terminal branches of the tree are colored according to the sampled location of the taxon at the tip. Internal branches are colored according to the most probable (modal) location of their parental node. Nodes with posterior probabilities (clade credibilities) of ≥ 0.95 have been labeled accordingly (in black). The probabilities of the modal locations of selected nodes are shown in blue (as percentages). The date of divergence (with 95% HPD in parentheses) of the 2009 Trinidad isolates is highlighted in gray. The histogram insert shows the posterior probabilities for the locations of the root nodes.

Trinidad, and south to Bolivia—although the link with Ecuador is again not statistically supported. Based on the BF estimates, the strongest epidemiological links are between Brazil and Venezuela, Brazil and Trinidad, and Peru and Bolivia.

The “among-region” analysis demonstrated the same gen-

eral trend of dispersal from Brazil and then Peru, with four source populations within Brazil (Para, North Central Amazonian Brazil, East Sub-Amazonian Brazil, and West Sub-Amazonian Brazil) and one in Peru (Peru South A, which includes Ayacucho and Cusco). When statistically well-supported links were considered, there was strong support for links within Peru (*in situ* evolution), but in the case of gene flow between Peru and other regions, there was only one relatively weakly supported link with Bolivia. In contrast, there are several well-supported linkages between Brazil and other regions.

Phylogenetic analysis of complete YFV structural polyprotein open reading frames. The prM/E gene regions of the Trinidad 2008–2009 isolates were identical, so isolate TVP11767 was randomly chosen as a representative of this

TABLE 4. Dates of divergence for prM/E gene sequences from YFV epizootics in Trinidad

Data set (<i>n</i>)	Date MRCA existed (95% HPD)
Trinidad 2009 isolates (5)	2004 (2000–2008)
Trinidad 2009 and 1995 isolates (6)	1986 (1974–1994)
Trinidad 1988–1989 isolates (4)	1978 (1968–1985)
Trinidad 1988–1989 and 1979 isolates (5)	1969 (1957–1978)

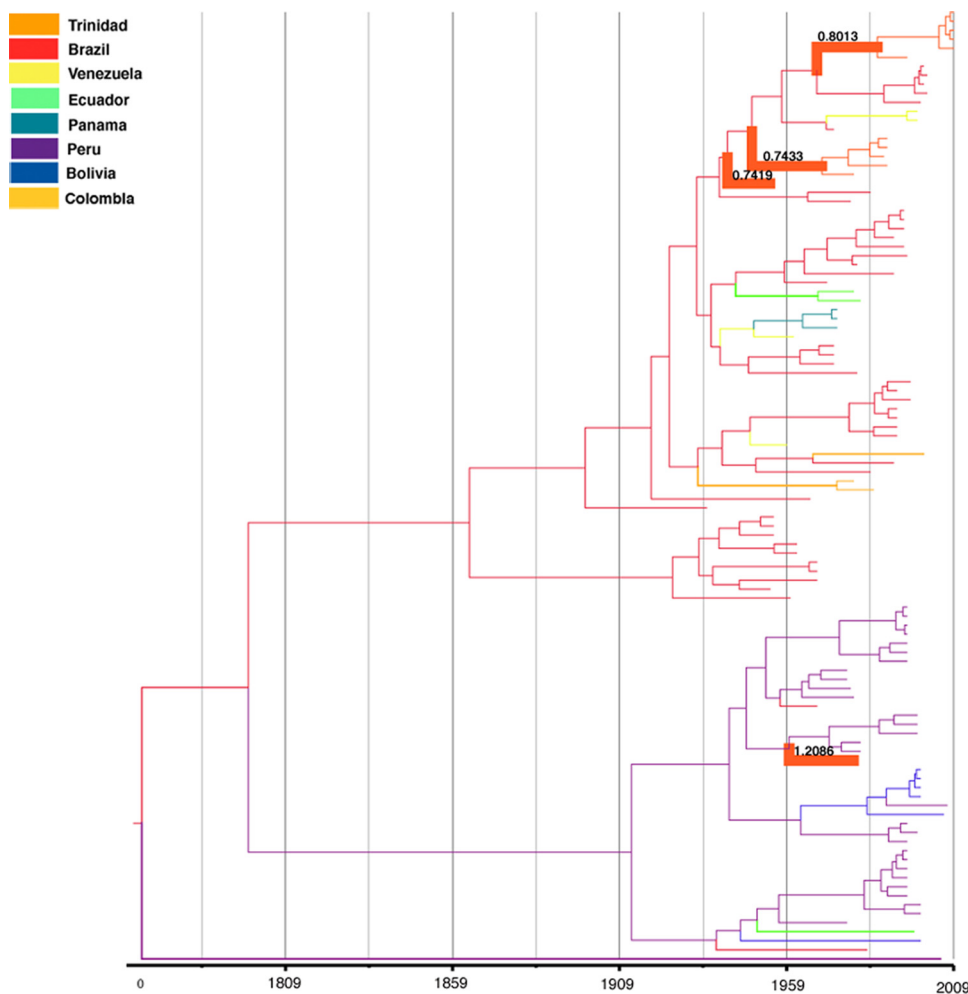


FIG. 2. MCC tree showing the inferred introductions into Trinidad from the mainland. Introductions are indicated by the wide orange branches. Terminal branches of the tree are colored according to the sampled location state of the taxon at the tip, and internal branches are colored according to the most probable (modal) geographic location state of the nodes supporting them. The branch thickness represents the median posterior jump count to Trinidad (the values are plotted along the four thick branches).

epizootic, and its complete genome was sequenced. There were 17 complete genomes available for comparison, and of these, 8 were reported as wild-type strains. These include the CAREC 788379 sequence, which was reported to be from an American isolate but is likely a contaminant (see Discussion). In the ML phylogeny, TVP11767 grouped separately from the African isolates and vaccine strains, with 100% bootstrap support (see Fig. S2 in the supplemental material). The amino acid and nucleotide sequence identities between each of the remaining 7 wild-type sequences and TVP11767 ranged from 92.7 to 96.6% and 78.7 to 85.5%, respectively (see Table S3 in the supplemental material). At the amino acid level, the NS3, NS1, NS5, envelope (E), and premembrane (prM) genes were the most conserved, and the capsid, NS2A, 2K, and NS2B genes were the most diverse (see Table S3 in the supplemental material).

DISCUSSION

YFV continues to be a significant public health problem in Africa and South America, and understanding the mainte-

nance and spread of the virus is integral to implementing surveillance strategies and prevention programs. In particular, the mechanisms underlying the apparently cyclical emergence and disappearance of YFV are not well understood. Using a powerful and robust Bayesian phylogeographic approach, we reconstructed the spatial and temporal spread of YFV in the Americas and investigated mechanisms of YFV maintenance, with particular reference to the island of Trinidad. Since Trinidad is small and separated from the mainland, YFV maintenance via *in situ* evolution between epizootics may readily be distinguished from reintroduction from neighboring regions.

Although the virus was apparently imported from Brazil into Trinidad on three occasions between 1954 and 2009 (Fig. 2), the posterior probabilities for node locations obtained using the Bayesian phylogeographic models provide strong statistical support for the 2008–2009 and 1988–1989 epizootics having arisen from viruses that existed within Trinidad since preceding epizootics (1995 and 1979, respectively) (Fig. 1). Given the small size of the island (4,828 km²) and the rapid rate at which the virus can spread across it (as illustrated by the pattern of monkey deaths in the most recent epizootic), it is unlikely that

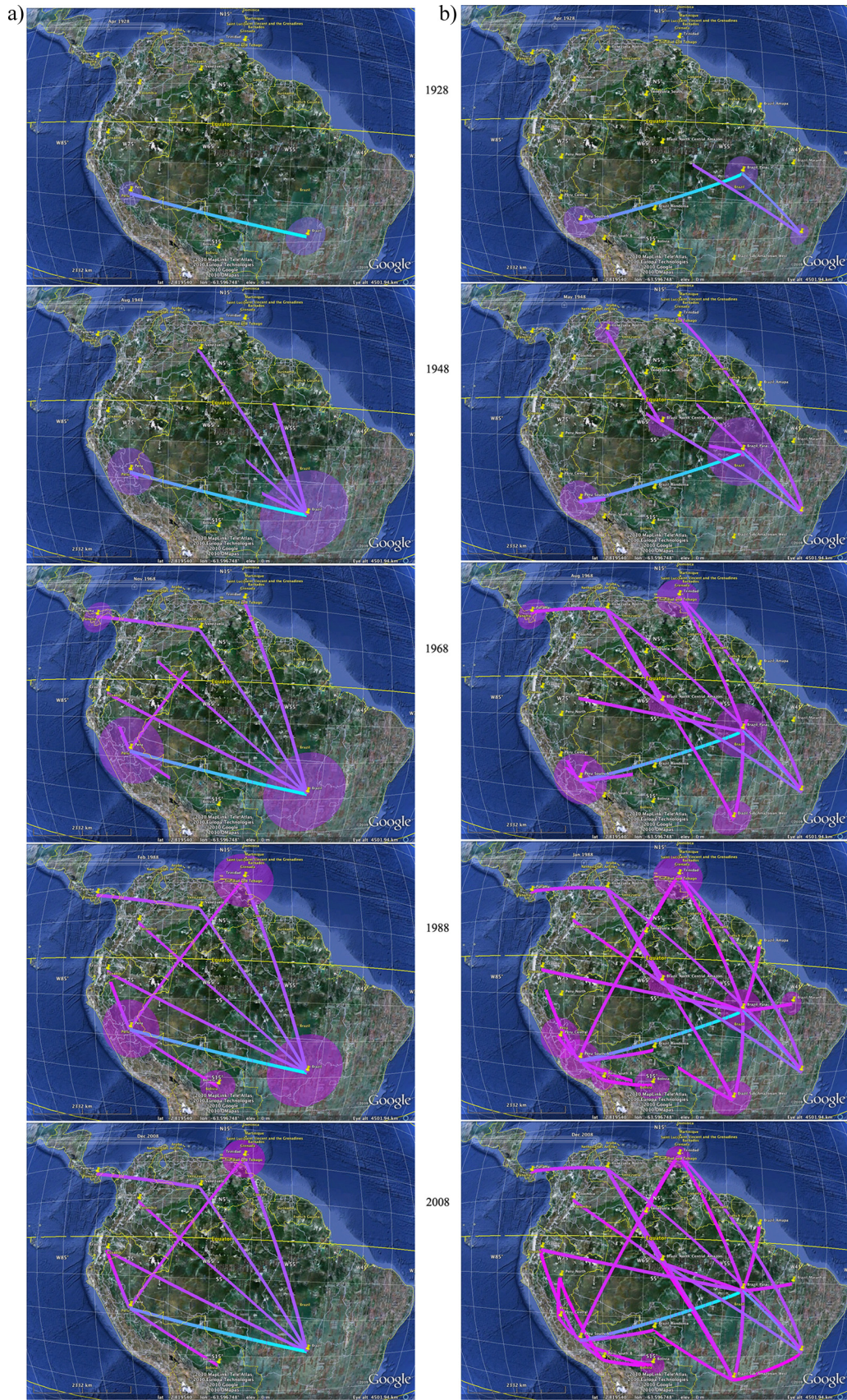


FIG. 3. Snapshots of dispersal patterns among countries (a) and among regions (b) at 20-year intervals from 1928 to 2008. Lines between locations represent branches in the MCC tree along which the relevant location transition occurs. Location circle diameters are proportional to the square root of the number of MCC branches maintaining a particular location state at each time point. The blue-purple color gradient indicates the relative ages of the transitions (older-recent). The maps are based on satellite pictures made available by Google Earth.

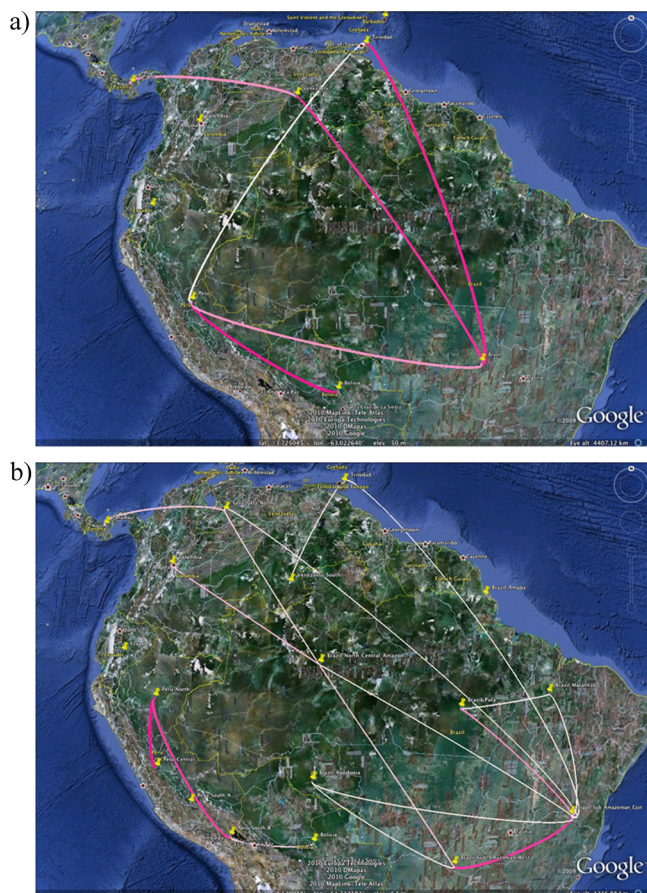


FIG. 4. BF test for significant nonzero rates. Rates supported by a BF of >3 are shown for the “among-country” (a) and “among-region” (b) analyses. The color and thickness of the line represent the relative strength by which the rates are supported; thin white lines and thick red lines suggest relatively weak and strong support, respectively. The maps are based on satellite pictures made available by Google Earth.

wandering epizootics occur continuously within Trinidad. Also, given the easy accessibility of Trinidad’s forests, the periods between reported epizootics more likely represent a lack of epizootic activity rather than periods during which epizootics go undetected. Thus, the most likely scenario is one of enzootic maintenance (*in situ* evolution) of the virus. Our results also suggest that the MRCA of the 2008–2009 Trinidad epizootic existed ~ 4.2 years prior to 2009 (95% HPD, 0.5 to 9.0 years). The upper limit for this estimate would date the MRCA to 2000, 5 years after the 1995 epizootic, suggesting that the MRCA for the 2009 epizootic is a descendant of the 1995 epizootic, as opposed to having been in circulation during the time of the 1995 outbreak. This would be consistent with a scenario where epizootics arise from viruses maintained in enzootic cycles.

Although there are clear instances of gene flow between regions within the Americas, as mentioned above, BaTS analysis detected strong structuring of the YFV phylogeny by country, suggesting that *in situ* evolution also plays an important role in the maintenance of YFV in the wider Americas. Additionally, the results of the BaTS analysis that considered the extent of population subdivision between individual countries

and all others as a group indicate that for four of the seven mainland countries sampled (i.e., Peru, Brazil, Panama, and Venezuela), the observed YFV genetic diversity is shaped primarily by *in situ* evolution as opposed to extensive migration. For Brazil and Peru, this is suggested by the tree topology (i.e., the existence of clades containing Brazilian or Peruvian isolates from time points separated by several years), and it was demonstrated previously for Peru through the use of Mantel’s test to assess the strength of correlation between genetic variability and geographic distribution (3). This scenario is now statistically supported by the estimated ancestral locations for the MRCAs of these clades and the nodes within them. Our Bayesian phylogeographic analyses also suggest the occurrence of *in situ* evolution within Colombia (the probability that the node supporting the 1979 and 1985 isolates existed in Colombia is 97%).

The spread of YFV inferred using “among-country” analysis and both 18-location and 28-location “among-region” analyses (28-location data are not shown) indicates that Brazil and Peru are primary source populations, with Brazil as the initial source of YFV diffusion. The identification of Brazil as the origin, despite the exclusion of the earliest sequence (Brazil 1935) from these analyses, and the corroborating evidence from the 28-location analysis both add to the credibility of our conclusions. It should also be noted that although many samples in our data set were obtained from Brazil and Peru, they comprise a very large part of the geographic area under study, which justifies an extensive sampling of these countries. Additionally, Fig. S1 in the supplemental material shows that the sampled sequences are more or less evenly distributed across the region of YFV endemicity.

All of the transmission pathways inferred were statistically supported, except for those with Ecuador and those between Brazil and Colombia (Fig. 4). There is strong statistical support for links within Peru (*in situ* evolution), but in the case of gene flow between Peru and other regions, there was only one relatively weakly supported link with neighboring Bolivia. In contrast, there are several well-supported linkages between Brazil and other regions. Interestingly, the majority are long-distance linkages between less-forested sub-Amazonian regions and other countries. This is consistent with long-distance spread of the virus via human movements rather than via nonhuman primates (9, 46). It may also reflect biases in sampling, with samples from urban centers presumably being overrepresented compared to those from the heavily forested regions where the virus is endemic. Such a combination of rapid long-distance movement and *in situ* evolution is consistent with our finding that distance-based proximity had little predictive power for among-location movement rates (i.e., there was a negligible difference in fit between the “equal rates” and “distance-informed” models).

The high degree of similarity observed among South American prME gene sequences is typical of YFV, which is genetically stable and evolves slowly in comparison to other arboviruses (4, 6, 38, 46, 52). It has been suggested that this may be a result of vertical transmission in mosquitoes, which may play an important role in the evolution of YFV in its enzootic setting (38). The ML tree inferred using all available YFV whole-genome sequences suggests that our Trinidad 2009 sequence (TVP11767) is the first complete genome to be re-

ported for a genotype I YFV, and it is highly divergent (15.2%) from the only other "South American" wild-type sequence available. The latter (CAREC 788379) was reported by Pisano et al. to have originated from a sylvatic mosquito (*Haemagogus spegazzini*) (34), but the close relationship of the reported sequence with those of the vaccine strain French neurotropic virus (FNV) and other vaccine strains suggests that a natural origin may be erroneous (due to labeling error or contamination). T1 mapping of CAREC 788379 by Deubel et al. (11) prior to the genomic sequencing by Pisano et al. provides further support for the notion that the sequence reported by Pisano et al. is that of a contaminant, since Deubel's results showed that CAREC 788379 generated a South American T1 map, while the sequence reported by Pisano et al. clusters with African isolates.

In summary, our results provide strong, statistically supported evidence for *in situ* evolution of YFV in many of the South American countries where this virus has been in circulation. YFV appears to be maintained locally in Trinidad for relatively long periods, and we hypothesize that epizootics emerge from viruses maintained in enzootic cycles. This would appear to be at odds with the observation of more or less synchronous epizootics in the Americas and begs the question of what mechanisms could account for this. There is currently no definitive answer, but the mechanisms are likely to involve complex interactions among multiple factors. Monath proposed that factors influencing YF activity, such as the presence of the virus, increases in the vector population, duration of the rainy season, humidity, temperature, and colonization with an absence of preventive strategies, occur similarly over wide geographic regions and could thus facilitate YFV emergence over wide areas in an approximately synchronous fashion (27). It is unclear whether enzootic maintenance of YFV results from persistent infection in monkeys, vertical transmission in mosquitoes, or maintenance in an alternative host, but among these, the most convincing evidence is provided for vertical transmission in mosquitoes (1, 2, 8, 12, 17, 20, 29, 38). Assuming that this is the case, then region-wide climatic changes that increase the number of transmission events between vertically infected mosquitoes and susceptible nonhuman primate populations could be responsible for simultaneous emergence of epizootic activity from enzootic cycles. In particular, temperature and rainfall can influence the availability of potential mosquito breeding sites and thus influence mosquito population sizes (37) and YFV dispersal (47). Additionally, increases in temperature shorten the extrinsic incubation period (i.e., the time between a mosquito taking an infected blood meal and being able to transmit the virus by bite) and can contribute to faster development of mosquito larvae (39).

Our findings are based on the best available data set for this type of analysis, as our data set includes all published YFV sequences for which geographic data are currently available, as well as newly derived sequences. However, our phylogeographic inferences relate to the populations sampled, and extrapolations to other regions should be made with caution. Further work is required to confirm the mechanism of enzootic maintenance of YFV in order to fully understand the role of ecological factors influencing emergence and dispersal. Trinidad may prove to be an ideal setting for such studies, as it is small and relatively isolated from other YFV-affected regions

yet has a mainland-like ecology, which will justify extrapolation of findings to the wider Americas. Finally, larger whole-genome data sets are clearly necessary for a more thorough understanding of YFV transmission and epidemiology, since the Trinidad 2009 genome reported in this study represents the only sequenced genome for a South American genotype I virus.

ACKNOWLEDGMENTS

This work was supported by grants from the Trinidad and Tobago Government Research Fund and the University of the West Indies, St. Augustine Campus, Research and Publications Fund (C.V.F.C. and A.J.A.), by NIH grant AI25489 (R.B.T. and S.C.W.), and by the John S. Dunn Foundation (S.C.W.). A.J.A. was supported by a scholarship from the University of the West Indies, St. Augustine Campus, Research and Publications Fund. P.L. was supported by a postdoctoral fellowship from the Fund for Scientific Research (FWO) Flanders. O.G.P. was supported by The Royal Society. M.A.S. was supported by NSF grant DMS 0856099 and NIH grant R01 GM086887.

A.J.A., C.V.F.C., and A.A.A. are members of the UWI St. Augustine Campus Tropical Medicine Cluster: Infectious Diseases.

We are grateful to Joe Parker for advice on the use of the BaTS program and to Orchid Allcock and Vernie Ramkisson for technical assistance. We are grateful to the TPHL, which provided CAREC with the monkey tissues used in this study.

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