

# Enzootic Transmission of Yellow Fever Virus, Venezuela

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Phylogenetic analysis of yellow fever virus (YFV) strains isolated from Venezuela strongly supports YFV maintenance in situ in Venezuela, with evidence of regionally independent evolution within the country. However, there is considerable YFV movement from Brazil to Venezuela and between Trinidad and Venezuela.

Yellow fever virus (YFV) is the prototype species for the genus *Flavivirus*. Historically, YFV is one of the most important human arboviral pathogens. It continues to cause large sporadic epidemics in Africa but typically emerges as epizootics among nonhuman primates in South America with or without associated human cases (1–5). YFV emergence is cyclical; outbreaks occur ≈7–10 years apart. Several phylogenetic studies have shown that YFV is locally maintained during these interepizootic periods in Peru (6), Brazil (7), and Trinidad (4). These studies also have indicated that the virus undergoes regionally independent evolution within some countries (6).

YFV has caused sporadic outbreaks in Venezuela; the most recently documented epizootic/epidemic occurred in 2005 (8). Although endemic to Venezuela, YFV has very rarely been isolated and characterized, and partial sequences have been determined only for 4 strains. Venezuela is located between Trinidad and Brazil, which have contributed major evidence for the enzootic maintenance of YFV in South America. Thus, sequencing Venezuelan YFV strains over a wide geographic area and temporal distribution might be valuable to test the hypothesis of local maintenance in Venezuela and to determine whether the virus moves regularly between Trinidad and Venezuela or between Venezuela and Brazil. Understanding the maintenance and spread of YFV in South America also is critical for developing effective surveillance and prevention strategies.

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We sequenced a prM/E gene fragment of 10 YFV isolates from 4 locations within Venezuela, spanning 6 years (2004–2010; Table 1). Additionally, we sequenced complete genomes for 5 representative isolates for comparison with 12 previously determined genomic sequences (4,9).

## The Study

The sporadic emergence of YFV in the Americas has been strongly associated with infection of red howler monkeys (*Alouatta seniculus*), which are particularly susceptible to disease. As exemplified in this study, nonhuman primate surveillance targeting this species remains an efficient strategy for monitoring enzootic YFV activity. Isolates made during a surveillance study aimed at investigating the ecology of infectious diseases in Venezuelan nonhuman primates were detected by cell culture and passaged once in Vero cells before sequencing, as previously described (4). Sequences were manually aligned in Se-AL (<http://tree.bio.ed.ac.uk/software/seal/>) and confirmed as nonrecombinant by using Recombination Detection Program (RDP4) (10). We obtained phylodynamic and phylogeographic estimates using Bayesian inference as implemented in BEAST v1.8.0 (11,12). We assessed the extent of geographic structuring using Bayesian tip-significance testing (13) based on the Markov chain Monte Carlo phylogenies estimated in BEAST. A Bayesian phylogeny was also inferred in Mr. Bayes (14) by using the general time reversible (GTR+I+ $\Gamma_4$ ) model for the complete open reading frame sequences.

Results of Bayesian tip-significance testing showed statistically significant geographic clustering among Venezuelan YFV strains. The association index, parsimony score, and maximum monophyletic clade statistics provided strong support ( $p < 0.01$ ) that strains from Venezuela cluster by location, suggesting that YFV is maintained for long periods within Venezuela. Similar results have been shown for Peru (6), Brazil, and Trinidad (4). The high posterior probabilities,  $> 0.99$ , observed at all nodes that delineate Venezuelan strains further support these conclusions (Figure 1).

We also found evidence of regionally independent evolution within Venezuela, as indicated by the existence of 2 phylogenetically distinct Venezuelan clades with posterior probabilities  $> 0.99$  (Figures 1, 2). The clade containing a 2004 strain (2A) from Guárico and a 2010 strain (10A) from Monagas represented all but one of the sequences from eastern Venezuela (i.e., east of Caracas; Table 1). The 7 other strains were collected on the western

**Table 1.** Yellow fever virus strains sequenced in the study and their metadata, Venezuela\*

Isolate ID	Source	Location	Year of collection	Passage history	GenBank accession no.
1A	Red howler monkey	Monagas	2004	Vero 2	KM388819
2A†	Red howler monkey	Guárico	2004	Vero 2	KM388817
3A	Red howler monkey	Portuguesa	2005	Vero 2	KM388820
4A	Red howler monkey	Portuguesa	2005	Vero 2	KM388821
5A	Human	Portuguesa	2005	Vero 2	KM388822
6A†	Human	Portuguesa	2005	Vero 2	KM388814
7A	Human	Portuguesa	2005	Vero 2	KM388823
8A†	Red howler monkey	Barinas	2006	Vero 2	KM388818
9A†	Red howler monkey	Apure	2007	Vero 2	KM388815
10A†	Red howler monkey	Monagas	2010	Vero 2	KM388816

\*ID, identification. Red howler monkey, *Alouatta seniculus* species.

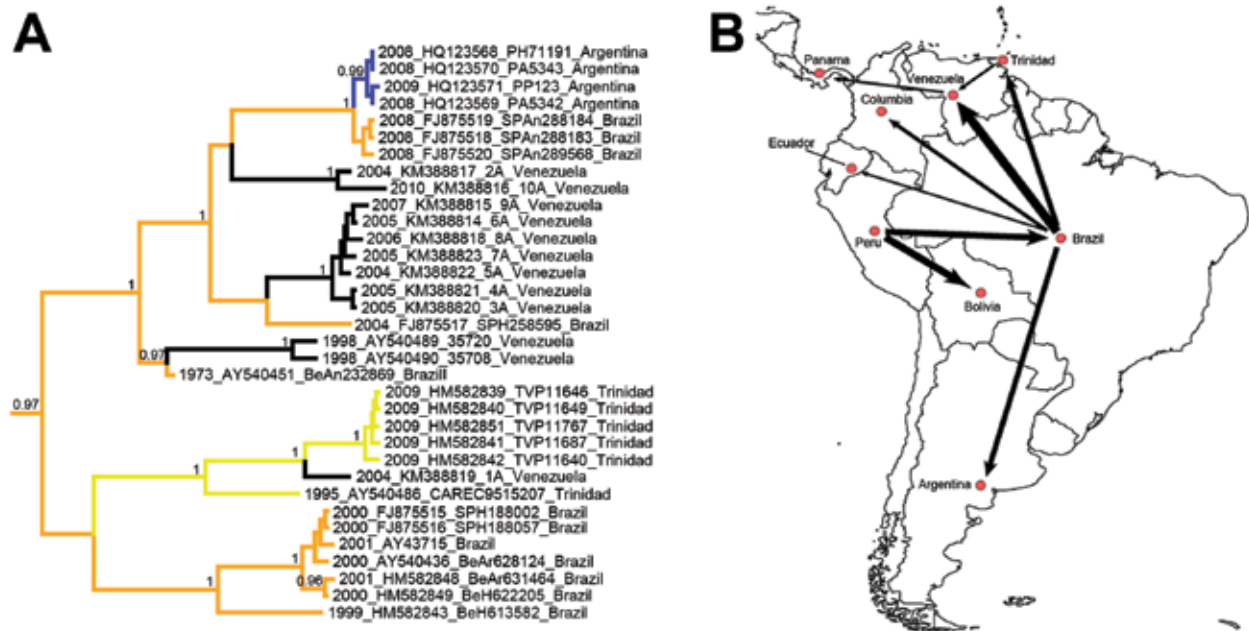
†Complete genome sequences determined.

side of Venezuela (including Portuguesa, Apure, and Barinas States) (Table 1). Two YFV strains were collected in 2004 from eastern and western Venezuela. Despite their nearly synchronous collections in 2004, these sequences fell into distinct clades in the maximum clade credibility (MCC) phylogeny, indicating population subdivision. Although we cannot rule out sampling bias, these data suggest in situ evolution of YFV in Venezuela and regionally independent evolution in distinct geographic foci within the country. The mechanism promoting this population subdivision among YFV strains is unclear and requires further investigation. Further studies on the ecology of the areas where these viruses were isolated might help explain the observed population subdivision.

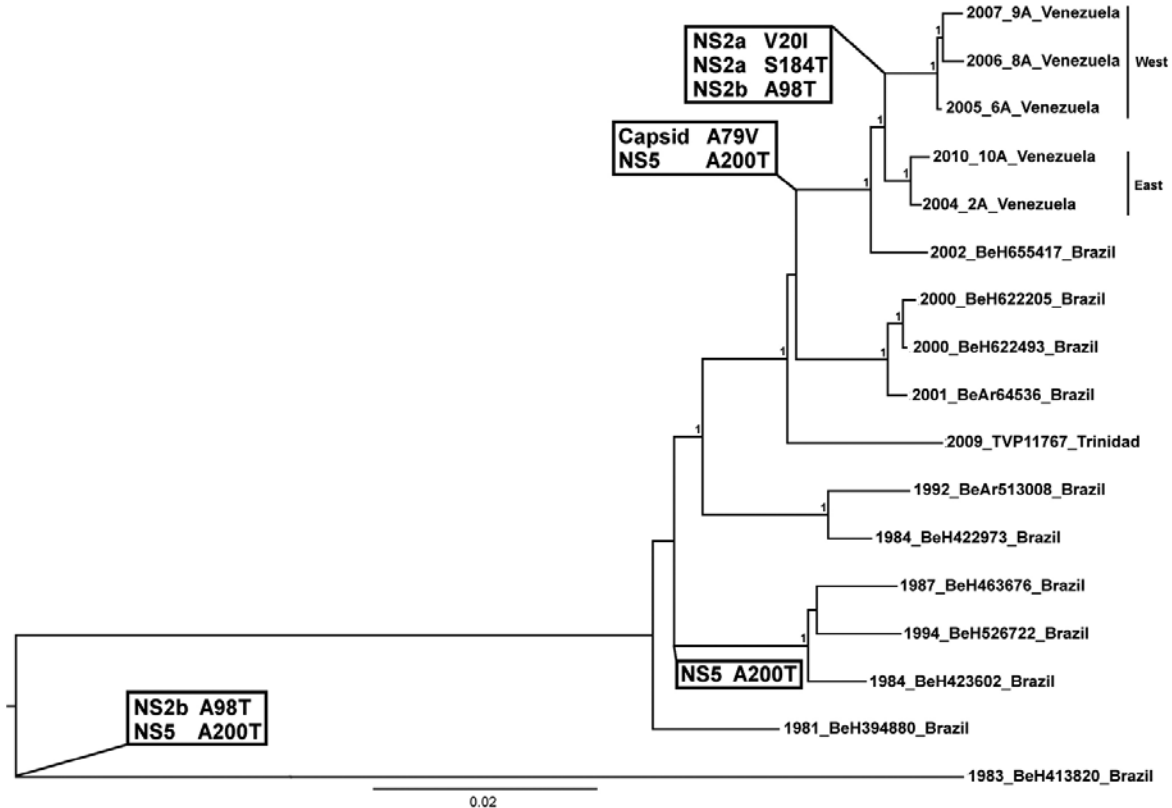
Although YFV had been maintained in situ for several years within Venezuela, our phylogeographic results indicated YFV movement between Brazil, Trinidad, and

Venezuela. Brazil is the major source of YFV introductions into Venezuela, accounting for introductions of Venezuelan strains sampled in 1959, 1961, 1998, and more recently (i.e., independent introductions in 2004 in eastern and western Venezuela; Figure 1). The basal location of a 2004 sequence from the Brazilian Amazon within the western Venezuelan 2004–2007 clade (Figure 1) suggests that the 2005 Venezuelan epizootic/epidemic was initiated by an imported progenitor from the Brazilian Amazon Basin that later evolved independently within Venezuela. Our estimate of the most recent common ancestor for the node containing the 2004–2007 Venezuelan sequences was 2001 (95% highest posterior density 1997–2003), suggesting that the ancestral lineage existed for  $\approx 3$  years in Venezuela before its detection in nonhuman primates in 2004.

The 2004 Venezuelan (1A) intermediary descendent between the 1995 and 2009 Trinidad sequences is noteworthy.



**Figure 1.** A) Magnified inset of the MCC phylogeny showing the tree topology for a subset of South American genotype I strains. B) Bayes factor (BF) test for significant nonzero rates indicating the statistical support for epidemiologically linked countries. Rates supported by a BF  $>5$  are shown. The thickness of the arrows represents the relative strength by which the rates are supported. The Technical Appendix (<http://wwwnc.cdc.gov/EID/article/21/1/14-0814-Techapp1.pdf>) presents the details of the 124 sequences used in this study.



**Figure 2.** Midpoint rooted Bayesian Markov chain Monte Carlo phylogeny based on yellow fever virus (YFV) complete open reading frame sequences. Numbers at nodes indicate posterior probabilities  $\geq 0.9$ . Eastern and western Venezuelan sequences are indicated. Substitutions resulting from nonsynonymous, synapomorphic mutations that define sequences in a clade/lineage are highlighted at relevant nodes. Two substitutions (NS2b A98T and NS5 A200T) occurred in earlier isolates from Brazil. The capsid A79V and NS5 A200T substitutions include the Brazilian isolate BeH655417, which lies directly basal to the Venezuela isolates. This indicates that these substitutions were probably present in the YFV progenitor when it was introduced into Venezuela. Furthermore, substitutions NS2a V10I, NS2a S184T, and NS2b A98T all appear to have arisen after YFV was introduced to Venezuela, further supporting enzootic YFV maintenance there. Taxon/tip labels include year of isolation, strain names and country where the virus was isolated. Scale bar indicates percentage of nucleotide sequence divergence.

This cluster of Trinidad isolates was previously used as evidence of enzootic YFV maintenance within Trinidad during interepizootic periods (4). Incorporating our new sequences now shows movement between Trinidad and eastern Venezuela. In our phylogeny, strain 1A is the sister lineage of the 2008–09 Trinidad epizootic strains, with the 1995 Trinidadian

isolate lying basal to these sequences, with high posterior probabilities (Figure 1). The position of the 2004 Venezuelan 1A sequence possibly reflects importation from Trinidad, which implies that enzootic YFV circulation in Trinidad is not isolated epidemiologically but gave rise to exportation of YFV to Venezuela.

**Table 2.** Nucleotide and amino acid divergence among individual YFV genes of 9 representative YFV strains compared to the TVP11767\* strain†

Genes	Strain, nucleotide (amino acid) divergence, %								
	BeH622205 (B, 2000)	BeAr51300 (B, 1992)	BeH423602 (B, 1984)	BeH413820 (B, 1983)	BeH394880 (B, 1981)	BeH655417 (B, 2002)	6A (V, 2005)	9A (V, 2007)	10A (V, 2010)
Capsid	98.3 (100)	98.0 (100)	97.2 (100)	91.2 (97.4)	96.3 (100)	97.2 (99.1)	97.2 (99.1)	97.2 (99.1)	96.6 (100)
PreM/M	97.7 (99.4)	96.7 (100)	91.2 (98.1)	97.3 (100)	97.3 (100)	96.2 (99.4)	97.7 (100)	97.7 (100)	97.7 (100)
E	98.2 (100)	96.3 (100)	89.6 (98.6)	96.9 (100)	97.5 (100)	96.6 (100)	97.2 (100)	97.0 (100)	97.6 (100)
NS1	97.9 (99.7)	97.3 (100)	90.5 (99.4)	97.5 (99.7)	98.1 (100)	96.8 (99.7)	98.7 (100)	98.4 (100)	98.6 (100)
NS2A&B	97.4 (100)	96.2 (99.4)	90.4 (99.4)	96.8 (100)	97.6 (100)	95.8 (99.1)	97.2 (100)	96.9 (99.4)	97.6 (100)
NS3	97.4 (100)	96.7 (100)	88.4 (100)	97.4 (100)	97.4 (100)	96.3 (100)	97.4 (100)	97.4 (100)	96.7 (100)
NS4A&B	98.2 (100)	96.8 (99.7)	90.3 (98.7)	97.1 (99.7)	97.8 (100)	97.1 (99.7)	98.1 (99.7)	98.0 (99.7)	98.2 (100)
NS5	98.3 (99.8)	96.6 (99.8)	91.1 (99.1)	96.8 (99.8)	98.0 (100)	96.6 (100)	98.4 (100)	98.1 (100)	98.2 (100)

\*First South American complete genome sequence to be published. TVP11767 was isolated in 2009 from an *Alouatta seniculus* specimen in Trinidad. †YFV, yellow fever virus; B, Brazil; V, Venezuela; M, membrane; E, envelope; NS, nonstructural.

In the absence of more dense sampling, whether bidirectional YFV movement occurs between Venezuela and Trinidad is unclear. Given the proximity and boating traffic between these countries, substantial mixing between their YFV populations would not be surprising. Isolation and sequencing of additional YFV isolates from eastern Venezuela are needed to further evaluate movement between Trinidad and Venezuela.

Complete genomes were sequenced for 5 representative Venezuelan YFV strains from eastern and western Venezuela (Table 1). Comparison of nucleotide and amino acid similarities showed a high degree of conservation across YFV genes (Table 2). The 9 strains selected for comparisons represent the full spectrum of known YFV genetic diversity (Table 2; Figure 2). The most diverse genes shared >97% aa sequence identity, with >99.9% aa sequence identity for most proteins, even though these strains were collected >30 years apart (Table 2). The Bayesian Markov chain Monte Carlo phylogeny based on complete open reading frame sequences strongly supported the inferred maximum clade credibility tree, with all Venezuelan sequences grouping together with strong posterior support. The eastern and western Venezuelan strains grouped separately, with strong support in all of our inferred phylogenies. A total of 5 substitutions delineated the Venezuelan sequences (Figure 2).

## Conclusions

Our phylogeographic analysis supports in situ evolution of YFV within Venezuela, as well as regionally independent evolution within the country. Brazil was identified as the major source of YFV introductions into Venezuela, and sequence analysis showed that considerable YFV movement may occur between Trinidad and Venezuela. Results of our Bayes factor test for non-zero rates also support the epidemiologic link between Venezuela, Brazil, and Trinidad (Figure 1, panel B). The sequences generated in our study fill a major gap in the geographic sampling of YFV.

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The GenBank accession numbers for the YFV sequences derived in this study are KM388814–KM388823.

Dr. Auguste is a postdoctoral fellow in the Department of Pathology, University of Texas Medical Branch, Galveston, Texas. His research interests include understanding the ecologic and evolutionary factors involved in emergence, dispersal, and maintenance of arboviruses and the genetic and structural characterization of novel arboviruses.

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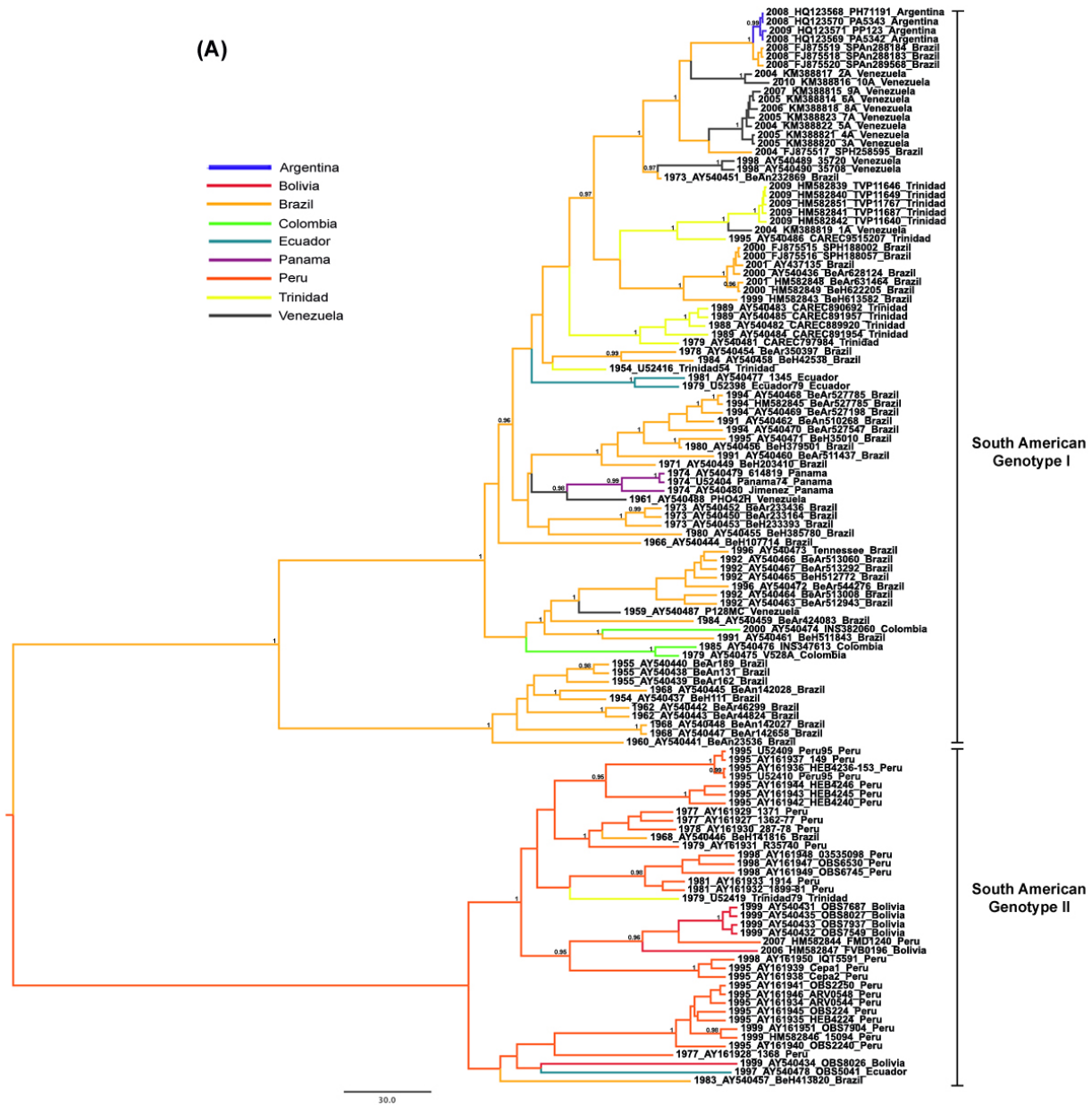
## Technical Appendix

Technical Appendix Table. Strains of yellow fever virus used in this study

GenBank accession no.	Strain name	Origin	Year	Location
AY540433	OBS7937	Santa Cruz	1999	Bolivia
AY540435	OBS8027	Santa Cruz	1999	Bolivia
AY540432	OBS7549	Santa Cruz	1999	Bolivia
AY540431	OBS7687	Santa Cruz	1999	Bolivia
HM582847	FVB0196	Unknown	2006	Bolivia
AY540434	OBS8026	La Paz	1999	Bolivia
AY540468	BeAr527785	Unknown	1994	Brazil
HM582845	BeAr527785	Unknown	1994	Brazil
AY540462	BeAn510268	Unknown	1991	Brazil
AY540451	BeAn232869	Unknown	1973	Brazil
AY540436	BeAr628124	Unknown	2000	Brazil
AY540458	BeH425381	Amapa	1984	Brazil
AY540471	BeH35010	Maranhao	1995	Brazil
AY540456	BeH379501	Maranhao	1980	Brazil
AY540444	BeH107714	Amazonas	1966	Brazil
AY540473	Tennessee	Manaus	1996	Brazil
AY540461	BeH511843	Roraima	1991	Brazil
AY540446	BeH141816	Para	1968	Brazil
AY540460	BeAr511437	Para	1991	Brazil
AY540470	BeAr527547	Para	1994	Brazil
AY540449	BeH203410	Para	1971	Brazil
AY540455	BeH385780	Para	1980	Brazil
AY540454	BeAr350397	Para	1978	Brazil
HM582843	BeH613582	Para	1999	Brazil
AY540459	BeAr424083	Para	1984	Brazil
AY540448	BeAn142027	Para	1968	Brazil
AY540447	BeAr142658	Para	1968	Brazil
AY540445	BeAn142028	Para	1968	Brazil
AY540437	BeH111	Para	1954	Brazil
AY540439	BeAr162	Para	1955	Brazil
AY540438	BeAN131	Para	1955	Brazil
AY540440	BeAr189	Para	1955	Brazil
AY540442	BeAr46299	Para	1962	Brazil
AY540443	BeAr44824	Para	1962	Brazil
AY540441	BeAn23536	Para	1960	Brazil
AY540457	BeH413820	Rondonia	1983	Brazil
AY540472	BeAr544276	Rondonia	1996	Brazil
AY540469	BeAr527198	Minas Gerais	1994	Brazil
HM582848	BeAr631464	Bahia	2001	Brazil
AY540450	BeAr233164	Goias	1973	Brazil
AY540452	BeAr233436	Goias	1973	Brazil
AY540453	BeH233393	Goias	1973	Brazil
HM582849	BeH622205	Goias	2000	Brazil
AY540464	BeAr513008	Mato Grosso do Sul	1992	Brazil
AY540463	BeAr512943	Mato Grosso do Sul	1992	Brazil
AY540465	BeH512772	Mato Grosso do Sul	1992	Brazil
AY540467	BeAr513292	Mato Grosso do Sul	1992	Brazil
AY540466	BeAr513060	Mato Grosso do Sul	1992	Brazil
AY437135	Not available	Unknown	2001	Brazil
AY540476	INS347613	Unknown	1985	Colombia
AY540475	V528A	Unknown	1979	Colombia
AY540474	INS382060	Unknown	2000	Colombia
AY540478	OBS5041	Pastaza	1997	Ecuador
U52398	Ecuador79	Unknown	1979	Ecuador

GenBank accession no.	Strain name	Origin	Year	Location
AY540477	1345	Unknown	1981	Ecuador
AY540480	Jimenez	Unknown	1974	Panama
U52404	Panama74	Unknown	1974	Panama
AY540479	614819	Unknown	1974	Panama
U52410	Peru95	Unknown	1995	Peru
U52409	Peru95	Unknown	1995	Peru
HM582844	FMD1240	Unknown	2007	Peru
HM582846	15094	Unknown	1999	Peru
AY161932	1899/81	Unknown	1981	Peru
AY161943	HEB4245	Junin	1995	Peru
AY161944	HEB4246	Junin	1995	Peru
AY161942	HEB4240	Junin	1995	Peru
AY161937	149	Pasco	1995	Peru
AY161940	OBS2240	Huanuco	1995	Peru
AY161945	OBS2243	Huanuco	1995	Peru
AY161941	OBS2250	Huanuco	1995	Peru
AY161936	HEB4236.153	Pasco	1995	Peru
AY161950	IQT5591	Loreto	1998	Peru
AY161946	ARV0548	S. Martin	1995	Peru
AY161934	ARVO544	S. Martin	1995	Peru
AY161935	HEB4224	S. Martin	1995	Peru
AY161951	OBS7904	S. Martin	1999	Peru
AY161949	OBS6745	Cusco	1998	Peru
AY161947	OBS6530	Cusco	1998	Peru
AY161948	3535098	Cusco	1998	Peru
AY161933	1914	Cusco	1981	Peru
AY161929	1371	Ayacucho	1977	Peru
AY161931	R35740	Ayacucho	1979	Peru
AY161928	1368	Ayacucho	1977	Peru
AY161927	1362/77	Ayacucho	1977	Peru
AY161930	287/78	Ayacucho	1978	Peru
AY161938	Cepa2	Puno	1995	Peru
AY161939	Cepa1	Puno	1995	Peru
U52419	Trinidad79	Unknown	1979	Trinidad
U52416	Trinidad54	Unknown	1954	Trinidad
AY540486	CAREC9515207	Unknown	1995	Trinidad
HM582851	TVP11767	Unknown	2009	Trinidad
HM582840	TVP11649	Unknown	2009	Trinidad
HM582841	TVP11687	Unknown	2009	Trinidad
HM582839	TVP11646	Unknown	2009	Trinidad
HM582842	TVP11640	Unknown	2009	Trinidad
AY540483	CAREC890692	Unknown	1989	Trinidad
AY540485	CAREC891957	Unknown	1989	Trinidad
AY540482	CAREC889920	Unknown	1988	Trinidad
AY540484	CAREC891954	Unknown	1989	Trinidad
AY540481	CAREC797984	Unknown	1979	Trinidad
AY540488	PHO42H	Tachira	1961	Venezuela
AY540487	P128MC	Cojedes	1959	Venezuela
AY540490	35708	Amazonas	1998	Venezuela
AY540489	35720	Amazonas	1998	Venezuela
FJ875515	SPH188002	Unknown	2000	Brazil
FJ875516	SPH188057	Unknown	2000	Brazil
FJ875517	SPH258595	Amazonas	2004	Brazil
FJ875518	SPAn288183	Unknown	2008	Brazil
FJ875519	SPAn288184	Unknown	2008	Brazil
FJ875520	SPAn289568	Unknown	2008	Brazil
HQ123568	PH71191	Unknown	2008	Argentina
HQ123569	PA5342	Unknown	2008	Argentina
HQ123570	PA5343	Unknown	2008	Argentina
HQ123571	PP123	Unknown	2009	Argentina
KM388819	1A	Monagas	2004	Venezuela
KM388817	2A	Guárico	2004	Venezuela
KM388820	3A	Portuguesa	2005	Venezuela
KM388821	4A	Portuguesa	2005	Venezuela
KM388822	5A	Portuguesa	2004	Venezuela
KM388814	6A	Portuguesa	2005	Venezuela
KM388823	7A	Portuguesa	2005	Venezuela
KM388818	8A	Barinas	2006	Venezuela
KM388815	9A	Apure	2007	Venezuela

GenBank accession no.	Strain name	Origin	Year	Location
KM388816	10A	Monagas	2010	Venezuela



Technical Appendix Figure. Bayesian maximum clade credibility (MCC) tree for YFV in the Americas based on 654 nt of the prM/E fragment. Taxon labels include year of isolation, GenBank accession number, 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 nodes. Nodes with posterior probabilities (clade credibilities)  $\geq 0.95$  are labeled accordingly in black. Scale bar indicates time in years.