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Molecular epidemiology of American/Asian genotype DENV-2 in Peru

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ABSTRACT

During the past decade, countries in South America have reported dengue hemorrhagic fever (DHF) associated with American/Asian genotype of dengue virus serotype 2 (DENV-2). DENV-2 strains have been associated with large outbreaks of dengue fever and DHF in numerous regions of Peru since the mid-1990s, but studies to address the origins, distribution, and genetic diversity of DENV-2 strains have been limited. To address this knowledge gap, we sequenced the envelope gene region of DENV-2 isolates from Peru, Ecuador, Paraguay, and Bolivia. Sequences were aligned and compared to a global sample of DENV-2 viruses. Phylogenetic analysis confirmed the circulation of two DENV-2 genotypes in Peru: American (prior to 2001) and American/Asian (2000 to present). American/Asian genotype variants can be classified into two lineages, and these were introduced into Peru from the north (Ecuador, Colombia, and/or Venezuela) and the east (Brazil and Bolivia). American/Asian lineage II replaced lineage I after 2009. We estimate the time to the most recent common ancestor for American/Asian DENV-2 genotype in the Americas was in 1980, and 1984 and 1989 for lineages I and II, respectively. In light of evidence for increased virulence of lineage II of American/Asian DENV-2, our results support the need for continuous monitoring for the emergence of new DENV genotypes that may be associated with severe disease.

1. Introduction

Dengue viruses (DENV) are members of the family *Flaviviridae*, genus *Flavivirus*, and are responsible for an estimated 50–100 million cases of febrile illness annually in tropical and subtropical areas (WHO, 2009). Disease can be caused by any of four different dengue virus serotypes (DENV-1, DENV-2, DENV-3, and DENV-4), and the clinical spectrum of disease ranges from asymptomatic infection to dengue fever (DF), dengue hemorrhagic fever (DHF), dengue shock syndrome (DSS), and death (Gubler, 1998). Epidemiological information links development of DHF with secondary DENV infections and also suggests that certain DENV strains are more virulent than others (OhAinle et al., 2011; Watts et al., 1999).

DENV-2 isolates can be divided into six genotypes based on the complete envelope (E) gene sequence: American/Asian, American, Cosmopolitan, Sylvatic, Asian I, and Asian II (Twiddy et al., 2002). The significant increase in the number of DHF/DSS cases in Latin America after 1981 has been largely attributed to the introduction of novel strains of DENV-2 from Southeast Asia, also termed subtype III (Rico-Hesse, 1990), now known as American/Asian. In 1995, Peru experienced a large dengue outbreak in Iquitos caused by American genotype DENV-2, yet, despite a high proportion of secondary infections, there was a striking absence of severe illness (Kochel et al., 2002; Watts et al., 1999). The first reported cases of dengue with hemorrhagic manifestations appeared during an outbreak (2000–2001) in northwestern Peru where multiple serotypes were circulating, including a DENV-2 strain that was initially identified as an Asian genotype by restriction fragment length polymorphism (RFLP) analysis (Montoya et al., 2003). Since 1995, DENV-2 has been circulating in different provinces, including in Amazonas, Cajamarca, San Martin, Loreto, Huánuco, Junín, Ucayali, Madre de Dios, Tumbes, Piura, Lambayeque, and La Libertad, but in the last few years DENV-2 transmission has intensified (DGE, 2012).

Several phylogenetic studies have documented the evolution of DENV-2 in different Latin American countries and the increase of DHF cases related to the introduction of new clades (Aquino et al., 2008; Bennett et al., 2006; Dettogni and Louro, 2012; Foster et al., 2004; McElroy et al., 2011; Mendez et al., 2012; Oliveira et al., 2010; Roca et al., 2009; Romano et al., 2010; Uzcategui et al., 2001). However, Peruvian strains from the last decade have not been thoroughly analyzed (Mamani et al., 2011). Little is known about the origins and evolutionary relationships among

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circulating strains. To characterize circulating strains and identify strain introductions into different geographic regions, we conducted a phylogenetic analysis based on E gene sequences. We utilized samples collected through an extensive clinic-based surveillance program established in sites in Peru, Bolivia, Ecuador, and Paraguay (Forshey et al., 2010). In addition, we estimated the time (in years) since American/Asian DENV-2 genotype strains entered in the Americas and since the main lineages diverged.

2. Materials and methods

2.1. Viruses, isolation and detection

DENV-2 was obtained from acute-phase sera collected from patients enrolled in a febrile surveillance program from 2000 to 2012 (Forshey et al., 2010). Samples were collected under human subjects protocols NMRC.2000.0006 and NMRCD.2010.0010, approved by the U.S. Naval Medical Research Center Institutional Review Board (Silver Spring, MD) and the U.S. Naval Medical Research Unit No. 6 Institutional Review Board (Lima, Peru), respectively. A total of 56 viral isolates were included in this study: 40 samples from Peru (9 from the northwest [Piura and Tumbes], 17 from the northeastern Amazon basin [Loreto], 3 from the eastern Amazon basin [Ucayali], 10 from the southeastern Amazon basin [Madre de Dios] and 1 from Junin), 2 from Ecuador, 11 from Bolivia and 3 from Paraguay. Collected sera were inoculated onto C6/36 mosquito cell monolayers, and viral isolates were identified as DENV-2 by indirect immunofluorescent assay (IFA) with serotype-specific monoclonal antibodies (Caceda and Kochel, 2007). The viral isolations were verified as DENV-2 by semi-nested reverse transcriptase polymerase chain reaction (RT-PCR) amplifications, as previously described (Lanciotti et al., 1992).

2.2. RNA extraction and sequencing

RNA was extracted from 140 µl of patient serum or supernatant from infected cells using QIAamp viral RNA Mini Kits (Qiagen), according to the manufacturer's instructions. RT-PCR was performed to amplify the entire 1485 bp E gene. The RT-PCR reaction mixture included 5 U of AMV reverse transcriptase (Promega), 2.5 U of GoTaq® polymerase (Promega), 1× PCR reaction buffer, 0.25 µM of each primer, 0.2 mM of dNTPs, 1.5 mM $MgCl_2$ and 5 µl of RNA. Reverse transcription occurred at 45 °C for 1 h. PCR amplifications consisted of 38 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 40 s), and extension (72 °C for 2 min) with a final extension at 72 °C for 10 min. Amplicons were purified with Centri-Sep columns (Invitrogen) and sequenced directly using the Big Dye terminator sequencing kit version 3.1 (Applied Biosystems) following the manufacturer's protocol. Primer sequences are provided in Table 1 (Wang et al., 2000). Sequencing was performed on a 3130 XL Genetic Analyzer (Applied Biosystems) platform, and sequences were analyzed using Sequencher (GeneCodes Corp.).

2.3. Phylogenetic analysis

ClustalW alignments (Thompson et al., 1994) were used to compare the Peru sequences generated here to publicly available GenBank sequences (http://www.ncbi.nlm.nih.gov) (Table 2). At least two sequences from other South American countries, such as Venezuela, Colombia, Ecuador, Brazil, Bolivia, and Paraguay, were included for each year over the last two decades. Phylogenetic trees were constructed using nucleotide sequences of the entire E gene, through Neighbor Joining (NJ), Maximum-likelihood (ML) and Bayesian methods. Sequences of American, Asian type 1, Asian type 2 and American/Asian genotype were included and sylvatic strains were used as the outgroup to root the trees (Table 3). We identified the Tamura-Nei model with gamma distributed plus invariant sites (TN93+G+I) as the best nucleotide substitution model for the E gene sequences, based on the Bayesian information criterion. The alignment, substitution model test, NJ, and ML analyses were conducted in MEGA5 (Tamura et al., 2011). Robustness of tree topology was assessed with 1000 bootstrap replicates. We considered nodes with bootstrap values >70% to be strongly supported in the phylogenetic analysis. Bayesian analysis was performed using MrBayes v3.2.1 (Ronquist and Huelsenbeck, 2003). For the substitution model, the General Time Reversible with 4 categories of gamma distributed rates plus invariable sites (GTR+G4+I) model with successive branch swapping was used. Four Markov Chain Monte Carlo (MCMC) chains were run for 10,000,000 generations, sampling every 1000 generations, with a burn-in of 2500. The consensus tree was visualized in FigTree 1.3.1.

2.4. Molecular dating analysis

The rate of nucleotide substitution and time to the most recent common ancestor for American/Asian genotype DENV-2 strains of South American origin were calculated using the Bayesian MCMC approach employed by BEAST ver. 1.6.1 (Drummond and Rambaut, 2007). The data was analyzed using the GTR+G4+I model and relaxed uncorrelated lognormal molecular clock. Constant population size tree prior was used to describe the DENV-2 demographic history. Analysis was done with 10 million MCMC generations, sampling every 1000 generations with a burn-in of 2500.

3. Results

To investigate the genetic relationships among DENV-2 strains from Peru and elsewhere in South America, we constructed phylogenetic trees using NJ, ML, and Bayesian methods. The consensus trees from all three methods showed the expected topology, classifying the sequences into the American (Tonga 74 and Peruvian strains), Asian type 1 (Thailand strains), Asian type 2 (New Guinea C and USA strains), and American/Asian (South America strains) genotypes. These results are in agreement with previously reported studies, which placed South American DENV-2 strains within American and American/Asian genotypes (Aquino et al., 2008; Romano et al., 2010; Twiddy et al., 2002; Uzcategui et al., 2001).

Table 1
Oligonucleotides used in this work.

Primers (genetic sense)	Sequence (5′→3′)		Reference
DEN2–835 (+)	CCAGGCTTTACCATAATGGC	RT-PCR & Sequencing	Romano et al. (2010)
DEN2–2420 (−)	CCAGCTGCACAACGCAACCAC	RT-PCR & Sequencing	
DEN2–1606 (+)	GCGGACACAAGGATCA	Sequencing	This study
DEN2–1752 (−)	CATCTGGATTTCTGTGGCC	Sequencing	

Table 2
List of DENV-2 isolates from South America included in the analysis.

GenBank accession no.	Strain name	Year of Isolation	Place	Country	Genotype	Lineage
JX051767	OBT1012/PER/00	2000	SULLANA-PIURA	PERU	AM/AS	I
JX051768	FSP011/PER/00	2000	TUMBES-TUMBES			I
JX051769	OBT1921/PER/01	2001	PIURA			I
JX051770	OBT2093/PER/01	2001	SULLANA-PIURA			I
JX051771	FSL699/PER/02	2002	IQUITOS-LORETO			I
JX051772	NFI052/PER/02	2002	IQUITOS-LORETO			I
JX051773	IQD2227/PER/02	2002	IQUITOS-LORETO			I
JX051774	IQD2856/PER/02	2002	PUNCHANA-LORETO			I
JX051775	IQD2005/PER/02	2002	IQUITOS-LORETO			I
JX051776	IQD2979/PER/02	2002	IQUITOS-LORETO			I
JX051777	FMD1346/PER/07	2007	TAHUAMANU-MADRE DE DIOS			I
JX051778	FMD1258/PER/07	2007	TAMBOPATA-MADRE DE DIOS			I
JX051779	FMD1337/PER/07	2007	TAMBOPATA-MADRE DE DIOS			I
JX051780	FMD2210/PER/08	2008	TAMBOPATA-MADRE DE DIOS			I
JX051782	FMD2276/PER/09	2009	TAMBOPATA-MADRE DE DIOS			I
JX051784	FMD2285/PER/09	2009	TAMBOPATA-MADRE DE DIOS			II
JX051781	FMD2303/PER/09	2009	TAHUAMANU-MADRE DE DIOS			I
JX051783	FMD2311/PER/09	2009	TAHUAMANU-MADRE DE DIOS			II
JX051785	FPI0337/PER/10	2010	IQUITOS-LORETO			II
JX051786	FPI1063/PER/10	2010	IQUITOS-LORETO			II
JX051787	FSL4823/PER/10	2010	ALTO AMAZONAS-LORETO			II
JX051788	FSL4874/PER/10	2010	LORETO			II
JX051789	NFI1166/PER/10	2010	MAYNAS-LORETO			II
JX051790	NFI1159/PER/10	2010	MAYNAS-LORETO			II
JX051791	FPY019/PER/10	2010	YURIMAGUAS-LORETO			II
JX051792	FPY016/PER/10	2010	YURIMAGUAS-LORETO			II
JX051793	FPI2645/PER/11	2011	IQUITOS-LORETO			II
JX051794	FPI2647/PER/11	2011	IQUITOS-LORETO			II
JX051795	FPM0149/PER/11	2011	PUERTO MALDONDO-MADRE DE DIOS			II
JX051796	FPM0152/PER/11	2011	PUERTO MALDONDO-MADRE DE DIOS			II
JX051797	MIS1312/PER/11	2011	TUMBES-TUMBES			II
JX051798	MIS1313/PER/11	2011	TUMBES-TUMBES			II
KC847991	FPU0356/PER/11	2011	PUCALLPA-UCAYALI			II
KC847992	FPT0754/PER/12	2012	TUMBES-TUMBES			II
KC847993	FPT0830/PER/12	2012	TUMBES-TUMBES			II
KC847994	FPU0395/PER/12	2012	PUCALLPA-UCAYALI			II
KC847995	FPU0574/PER/12	2012	PUCALLPA-UCAYALI			II
KC847996	FPI3922/PER/12	2012	IQUITOS-LORETO			II
JX051799	OBS9011/EC/00	2000	GUAYAQUIL-GUAYAS	ECUADOR		I
JX051800	OBS8883/EC/00	2000	BABAHOYO-LOS RIOS			I
JX051801	FSB407/BOL/03	2003	ANDRES IBAÑEZ-SANTA CRUZ	BOLIVIA		II
JX051808	FSB1193/BOL/06	2006	ANDRES IBAÑEZ-SANTA CRUZ			II
JX051806	FSB2080/BOL/07	2007	DIAZ-BENI			I
JX051809	FSB2033/BOL/07	2007	CERCADO-BENI			II
JX051802	FSB1721/BOL/07	2007	ANDRES IBAÑEZ-SANTA CRUZ			II
JX051803	FSB1704/BOL/07	2007	SANTA CRUZ			II
JX051804	FSB1671/BOL/07	2007	SANTA CRUZ			II
JX051805	FCB606/BOL/07	2007	CONCEPCION-SANTA CRUZ			II
JX051807	FSB3514/BOL/10	2010	CERCADO-BENI			II
JX051810	FSB3528/BOL/10	2010	CERCADO-BENI			II
JX051811	FSB3355/BOL/10	2010	CERCADO-BENI			II
JX051812	FPA1588/PAR/10	2010	ASUNCION	PARAGUAY		II
JX051814	FPA1597/PAR/10	2010	ASUNCION			II
JX051813	FPA2341/PAR/10	2010	ALTO PARANA			II
HQ012538	BR39145/RJ/90	1990	RIO DE JANEIRO	BRAZIL	AM/AS	I
HQ012534	BR42727/RJ/91	1991	RIO DE JANEIRO			I
HQ012535	BR48622/CE/94	1994				I
HQ012511	BR52477/RJ/95	1995	RIO DE JANEIRO			I
GQ368158	1998/BR/63415	1998	RIO DE JANEIRO			I
GQ368159	1998/BR/63428	1998	RIO DE JANEIRO			I
HQ012537	BR64905/RJ/99	1999	RIO DE JANEIRO			I
FJ850072	DENV-2/BR/BID-V2376/2000	2000	NORTHERN			I
FJ850074	DENV-2/BR/BID-V2379/2001	2001	NORTHERN			II
HQ012521	BR72308/RJ/2001	2001	RIO DE JANEIRO			I
FJ850076	DENV-2/BR/BID-V2382/2002	2002	NORTHERN			II
HQ012523	BR76012/ES/2002	2002	ESPIRITU SANTO			I
FJ850078	DENV-2/BR/BID-V2386/2003	2003	NORTHERN			II
HQ012524	BR77395/ES/2003	2003	ESPIRITU SANTO			I
FJ850082	DENV-2/BR/BID-V2390/2004	2004	NORTHERN			II
FJ850085	DENV-2/BR/BID-V2393/2005	2005	NORTHERN			II
FJ850088	DENV-2/BR/BID-V2396/2006	2006	NORTHERN			II
FJ850091	DENV-2/BR/BID-V2399/2007	2007	NORTHERN			II
GQ368164	2007/BR/88034	2007	RIO DE JANEIRO			II
GQ368162	2007/BR/87183	2007	RIO DE JANEIRO			II

Table 2 (*continued*)

GenBank accession no.	Strain name	Year of Isolation	Place	Country	Genotype	Lineage
HQ012526	BR88034/RJ/2007	2007	RIO DE JANEIRO			II
GQ368172	2008/BR/337-08	2008	RIO DE JANEIRO			II
GQ368167	2008/BR/258	2008	RIO DE JANEIRO			II
HM181971	DENV-2/BR/BID-V3637/2008	2008	SAO PAULO			II
HQ012530	BR0145/ES/2009	2009	ESPIRITU SANTO			II
HQ012529	BR0066/BA/2009	2009	BAHIA			II
HQ012532	BR0199/RJ/2010	2010	RIO DE JANEIRO			II
HQ012531	BR0023/RJ/2010	2010	RIO DE JANEIRO			II
AF100466	MARA4	1990		VENEZUELA		I
AY158329	MARA3	1990				I
GQ868598	DENV-2/VE/BID-V3365/1991	1991				I
GQ868595	DENV-2/VE/BID-V3362/1991	1991				I
EU687220	DENV-2/VE/BID-V1456/1996	1996	ARAGUA			I
EU726775	DENV-2/VE/BID-V1457/1996	1996	ARAGUA			II
FJ898465	DENV-2/VE/BID-V2941/1998	1998	ARAGUA			I
AF398108	LARD4341	1999				II
AF398106	LARD3146	1999				I
FJ898466	DENV-2/VE/BID-V2942/2000	2000	ARAGUA			II
AF398113	LARD6045	2000				II
JN819408	DENV-2/VE/BID-V2161/2001	2001	ARAGUA			II
FJ639734	DENV-2/VE/BID-V2160/2003	2003	ARAGUA			II
FJ639783	DENV-2/VE/BID-V2216/2003	2003	ARAGUA			II
FJ850112	DENV-2/VE/BID-V2424/2004	2004	CARACAS			II
FJ639788	DENV-2/VE/BID-V2221/2004	2004	ARAGUA			II
FJ898467	DENV-2/VE/BID-V2944/2005	2005	ARAGUA			II
FJ639733	DENV-2/VE/BID-V2159/2005	2005	ARAGUA			II
FJ639822	DENV-2/VE/BID-V2262/2006	2006	ARAGUA			II
GQ868641	DENV-2/VE/BID-V1144/2007	2007	ARAGUA			II
FJ850105	DENV-2/VE/BID-V2470/2007	2007	ARAGUA			II
FJ850107	DENV-2/VE/BID-V2477/2008	2008	ARAGUA			II
AF363078	LARD1701	2008				II
FJ850106	DENV-2/VE/BID-V2476/2008	2008	ARAGUA			II
AF363092	LARD2995	2008				II
AY577431	360281	1992		COLOMBIA		I
DQ364512	COL_A_93	1993				I
DQ364497	COL_97	1997				I
GQ868552	DENV-2/CO/BID-V3368/1998	1998	SANTANDER			I
GQ868553	DENV-2/CO/BID-V3369/1999	1999	SANTANDER			II
GQ868554	DENV-2/CO/BID-V3370/2004	2004	SANTANDER			II
FJ024477	DENV-2/CO/BID-V1603/2004	2004	ANTIOQUIA			II
FJ182012	DENV-2/CO/BID-V1597/2005	2005	GUAVIARE			II
JF804029	CI/DB016/2007	2007				II
GU131947	DENV-2/CO/BID-V3374/2007	2007	SANTANDER			II
EU045311	D2PY-04/01	2001		PARAGUAY		II
EU045312	D2PY-21/05	2005				II
EU045313	D2PY-22/05	2005				I
AY484649	EC2000A	2000		ECUADOR		I
AY484651	EC2000B	2000				I
AY484611	CDC337325_BOL1997	1997		BOLIVIA		I
X54319	TONGA 74	1974		TONGA	AMERICAN	
DQ917242	IQT 1950	1995	LORETO	PERU		
JX051765	OBS7584/PER/99	1999	JUNIN			
JX051766	OBS9638/PER/00	2000	SULLANA-PIURA			
AF038403	NEW GUINEA C 44	1944		NEW GUINEA	ASIAN TYPE 2	
AF204178	43	1987	GUANGXI	CHINA		
JN796245	DENV-2/US/BID-V5055/2008	2008	CALIFORNIA	USA		
JF730055	DENV-2/US/BID-V5414/2009	2009	CALIFORNIA			
HQ541799	DENV-2/US/BID-V4825/2010	2010	CALIFORNIA			
DQ181806	ThD2_0038_74	1974	BANGKOK	THAILAND	ASIAN TYPE 1	
DQ181805	ThD2_0168_79	1979	BANGKOK			
X15434	M1	1987		MALAYSIA		
AF231717	P8 1047 70	1970		MALAYSIA	SYLVATIC	
AF231718	DAKAr578 80	1980		IVORY COAST		
AF231719	PM33974 81	1981		GUINEA		
AF231720	DAKHD10674	1970		SENEGAL		

The sequences obtained are in bold.

Similarly, Peruvian DENV-2 isolates clustered within the American and American/Asian genotypes. The American genotype circulated in Peru between 1995 and 2000. Since 2000, the American/Asian genotype has been the only genotype detected, with the exception of a brief period of time when both the American and the American/Asian genotypes co-circulated in northwestern Peru.

Sequences from Peru and elsewhere in South America that grouped into the American/Asian genotype were divided in two clearly defined lineages, termed lineages I and II, which were supported by high bootstrap values (>70%) and posterior probability (>0.8) (Fig. 1A). For Peruvian sequences, strains from 2000 to 2009 grouped into lineage I, while the strains from 2009 to 2012

Table 3
Amino acid comparison of American/Asian DENV-2 genotype from South America.

		61	91	118	129	131	160	170	203	340	380	432	462	
	DQ364484 JAMAICA 83	I	L	M	V	L	K	I	D	M	I	I	I	CLADE
LINEAGE 1	BRAZIL 90, 91, 94, 95, 00	.	I	.	.	.	E	.	E	.	.	.	.	B
	BOLIVIA 97	.	I	.	.	.	E	.	E	.	.	.	.	
	LORETO-PERU 02	.	I	.	.	.	E	.	E	.	.	.	.	
	BRAZIL 98-03	.	I	.	.	.	.	.	E	.	.	.	.	
	PARAGUAY 05	.	I	.	.	.	.	.	E	.	.	.	.	
	BOLIVIA 07	.	I	.	.	.	.	.	E	.	.	.	.	
	MADRE DE DIOS-PERU 07-09	.	I	.	.	.	.	.	E	.	.	.	.	
	VENEZUELA 90-99	.	I	.	.	.	.	.	E	.	.	.	.	A
	COLOMBIA 92-98	.	I	.	.	.	.	.	E	.	.	.	.	
	ECUADOR 00	.	I	K	.	.	.	.	E	.	.	L	.	
	TUMBES, PIURA-PERU 00, 01	.	I	K	.	.	.	.	E	.	.	L	.	
LINEAGE 2	BRAZIL 01-06	V	I	.	.	Q	.	.	.	.	.	.	.	C
	PARAGUAY 01-05	V	I	.	.	Q	.	.	.	.	.	.	.	
	BOLIVIA 03-07	V	I	.	.	Q	.	.	.	.	.	.	.	
	VENEZUELA 96-08	.	I	.	.	Q	.	.	.	T	.	.	.	E
	COLOMBIA 99-04-07	.	I	.	.	Q	.	.	.	T	.	.	.	
	TUMBES-PERU 11-12	.	I	.	.	Q	.	T	.	T	.	.	.	
	VENEZUELA 03-08	.	I	.	.	Q	.	.	.	T	.	.	V	D
	COLOMBIA 04-07	.	I	.	.	Q	.	.	.	T	.	.	V	
	BRAZIL 07-10	.	I	.	I	Q	.	T	.	T	V	.	.	F
	MADRE DE DIOS-PERU 09-10	.	I	.	I	Q	.	T	.	T	V	.	.	
	LORETO 10-12	.	I	.	I	Q	.	T	.	T	V	.	.	
	BOLIVIA 10	.	I	.	I	Q	.	T	.	T	V	.	.	
	PARAGUAY 10	.	I	.	I	Q	.	T	.	T	V	.	.	

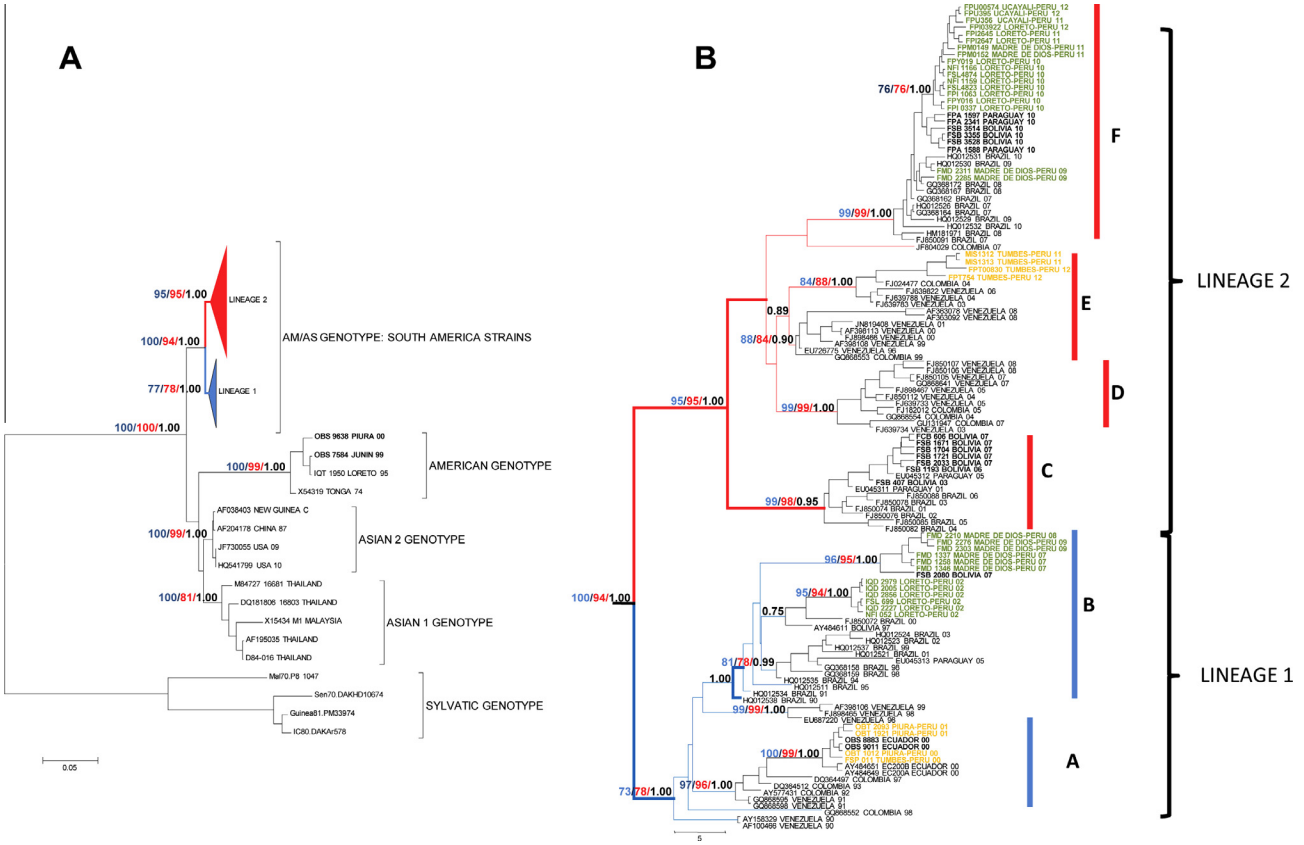

Fig. 1. Bayesian Markov chain Monte Carlo (MCMC) tree based on envelope gene sequences of DENV-2. (A) Phylogenetic tree depicting the relationship between DENV-2 viruses from Peru and other countries in South America. Members of five of the six reported genotypes were included. Sylvatic genotype strains were considered as the outgroup. (B) Bayesian tree showing in detail the relationships between South American strains collected from 1990 to 2012. NJ bootstrap values (blue), ML bootstrap values (red), and Bayesian posterior probability values (black) are shown above each principal node. Only bootstrap values >70% and posterior probabilities >0.85 are shown. Sequences generated in this study are shown in bold. Sequences from northeastern and southeastern Peru are shown in green, while sequences from northwestern Peru are shown in yellow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

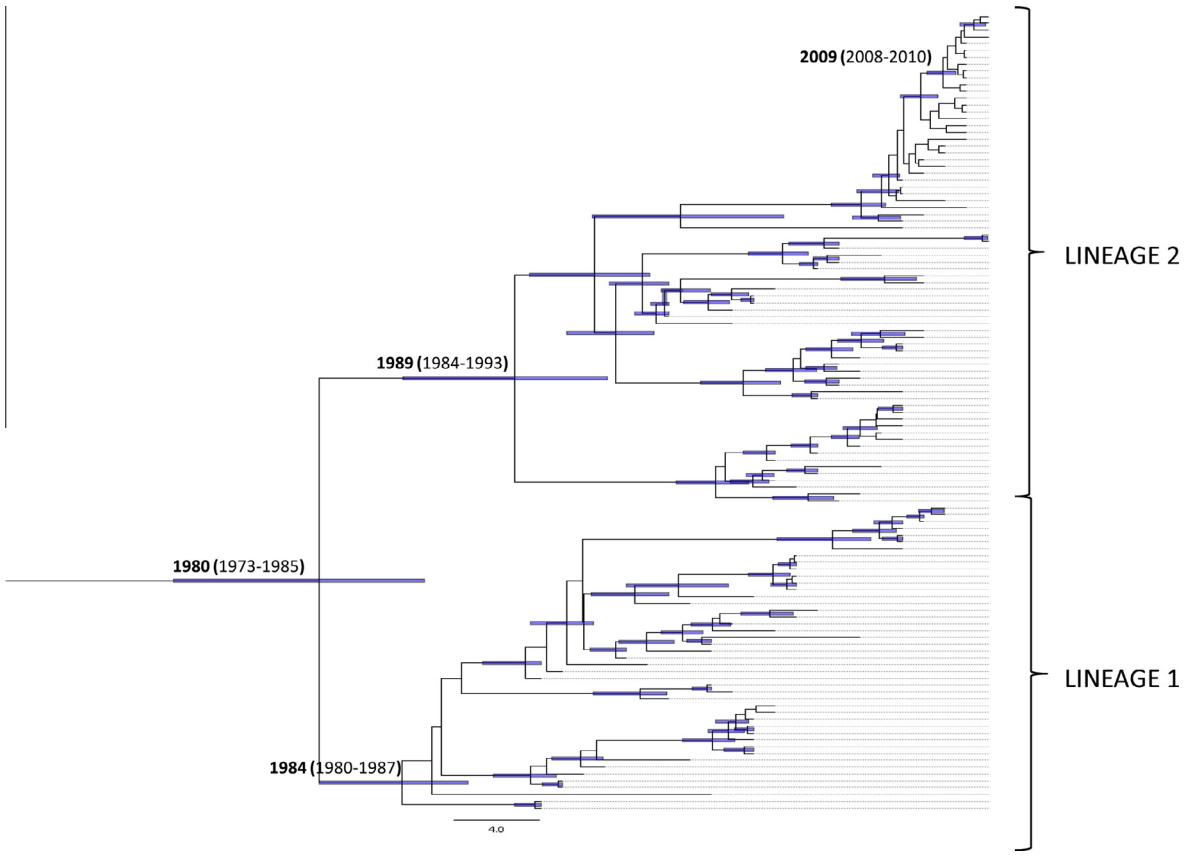

Fig. 2. The time to the most recent common ancestor of the more recent lineages of American/Asian genotype of DENV-2. The mean time is shown in each principal node (mean and 95% HPD). The 95% highest posterior density for each node age is shown as a blue bar.

were included into lineage II (Fig. 1B). However, strains belonging to lineage I were detected in South America between 1990 and 2009, and those belonging to lineage II were detected from 1996 onwards (Fig. 1B).

Different clades within each lineage were identified; specifically, two clades (A and B) belonging to lineage I and four clades (C–F) belonging to lineage II (Fig. 1B). Peruvian strains were distributed within lineage I (clades A and B) and lineage II (clades E and F). The strains from northwestern Peru in 2000–2001 were related to Ecuadorian (2000), Venezuelan (1991), and Colombian (1992, 1993, and 1997) strains (lineage I-clade A). The lineage I-clade B strains circulating from 2002 to 2009 in the Amazon region were related to DENV-2 (Fig. 1B) strains from Brazil (1990–2003), Paraguay (2005) and Bolivia (1997, 2007). Lineage II-clade F strains from Madre de Dios, Ucayali and Loreto between 2009 and 2012 grouped with Brazilian, Bolivian, and Paraguayan DENV-2 strains circulating between 2007 and 2010 (Fig. 1B). In contrast, strains from Tumbes during the same period (2011–2012) belonged to lineage II-clade E with Colombian (2004) and Venezuelan (2003–2006) strains.

To identify amino acid residues that could distinguish American/Asian genotype strains, we compared amino acid sequences of the E gene product. Several amino acid residues varied between American/Asian genotype strains and the Jamaica 83 strain (Table 3). Additionally, amino acid sites 131 (lineage I: L (hydrophobic); lineage II: Q (polar uncharged)), 203 (lineage I: E (polar charged); lineage II: D (polar charged)) and 340 (lineage I: M (hydrophobic); lineage II: T (polar uncharged)) varied between the two American/Asian genotype lineages.

To better understand the epidemiology of the American/Asian genotype DENV-2 from Peru and South America, we calculated the time to the most recent common ancestor for the genotype as a whole and separately for the two lineages (Fig. 2). We estimated that the most recent common ancestor for South American strains of the American/Asian genotype was in 1980 (95% highest posterior density (HPD): 1973–1985). For lineage I, the most recent common ancestor was approximately in 1984 (95% HPD: 1980–1987), while for lineage II, the most recent common ancestor was approximately in 1989 (95% HPD: 1984–1993). In the case of Peruvian strains responsible for a large and severe dengue outbreak in northeastern Peru (2010/2011 outbreak in Loreto), sequence analysis traced the most recent common ancestor to 2009 (95% HPD: 2008–2010).

4. Discussion

We demonstrated the circulation of two DENV-2 genotypes in Peru, American and American/Asian. The American genotype was first detected in 1995 and continued to circulate until 2000. In 2000, the first isolates of the American/Asian genotype were identified, and the American genotype appears to have become extinct in Peru. The emergence of the American/Asian DENV-2 genotype coincided with an increase in severity of cases in Peru, as had been reported for other countries in the Americas with DENV-2 strains from Asia (Bennett et al., 2006; Foster et al., 2004; Rico-Hesse et al., 1997; Uzcategui et al., 2001). As previously reported (Mamani et al., 2011), the American/Asian genotype isolates associated with a large epidemic of DHF in northeastern Peru in 2010 were distinct from DENV-2 isolates previously circulating in the region.

Since 2000, the evolution of American/Asian genotype DENV-2 in Peru can be characterized by the introduction of four different

Fig. 3. Map of potential routes of DENV-2 introductions into Peru. Peruvian provinces with isolates of lineage II of the American/Asian genotype of DENV-2 are shown in yellow (lineage II-clade E) and green (lineage II-clade F). Two proposed routes of spread for lineage II are shown. Lineage II-clade E was introduced into northern Peru (Tumbes and Piura) from northern South America (Venezuela/Colombia). Furthermore, lineage II-clade F was initially introduced into southern Peru from Bolivia or Brazil and subsequently spread to the northeastern jungle region (Loreto and Ucayali). Countries other than Peru where we collected isolates for this analysis are shaded in gray. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

clades distributed within lineages I and II. We estimated that the American/Asian genotype emerged in the Americas 33 years ago, suggesting that the genotype appeared a year before the first DHF cases were reported in Latin America (Cuba and Jamaica in 1981) (Gubler, 1998) and that the common ancestors of lineage I and II emerged 29 and 24 years ago, respectively. These dates are consistent with previous reports of American/Asian DENV-2 in the Americas (Anez et al., 2011; Carrington et al., 2005; Foster et al., 2004; Romano et al., 2010). The genetic divergence among strains suggest that lineage I and lineage II were both independently introduced into northwestern Peru (lineage I-clade A in 2000; lineage II-clade E in 2011) and eastern Peru (lineage I-clade B in 2002; lineage II-clade F in 2009).

Routes of entry into Peru have been postulated for other DENV serotypes. Prior phylogenetic analysis conducted on DENV-3 suggested that this virus was introduced into northwestern Peru from Ecuador, but the dissemination route to Loreto in northeastern Peru was not established (Kochel et al., 2008). Phylogenetic analysis of DENV-4 also supported an introduction event into northwestern Peru and Ecuador from the northern region of South America before 2006, and a subsequent introduction from northwestern Peru into the northeastern department of Loreto (Forshey et al., 2009). Similarly, our results suggest that lineage I-clade A (2000/2001) and lineage II-clade E (2011/2012) were also introduced using the same route from northern region of South America (Fig. 3). Although only Ecuadorian sequences from lineage I-clade A in 2000 were available for analysis, we cannot rule out the possibility that lineage II-clade E was also introduced via Ecuador from Colombia or Venezuela, and later into northern Peru, consistent with previous findings for DENV-3 and DENV-4. In contrast, we found that the lineage II-clade F strains circulating in northeastern Peru in 2010 were similar to those from southeastern Peru isolated in 2009 and most closely related to strains from Paraguay, Bolivia, and Brazil, rather than countries to the north, supporting another introduction event from the southeastern region of South America.

Introductions of novel clades are sometimes associated with a shift in dominant serotype. For example, during the introduction of new DENV-2 clades in Paraguay (lineage II-clade C) and Brazil (lineage II-clade F) in 2005 and 2008, respectively, the dominant serotype (DENV-3) was displaced by DENV-2 (Aquino et al., 2008; Oliveira et al., 2010). Likewise, introduction of American/Asian genotype strains was associated with serotype and lineage replacements. Lineage II (clade F) was introduced into southeastern Peru (Madre de Dios), likely from Brazil, in mid-2009 (Fig. 3). Lineage I and lineage II co-circulated in southeastern Peru for a short period, followed by lineage I displacement in 2010. Lineage II (clade F) also displaced DENV-4 as the dominant serotype in Loreto in 2011. These data support the notion that the introduction of new DENV-2 variants belonging to lineage II (clade F) could be associated with the shift of dominant serotypes and is congruent with the report from Brazil (Oliveira et al., 2010).

Several amino acid polymorphisms were identified that could discriminate between American and American/Asian genotype and which might have important phenotypic and epidemiologic

consequences. Amino acid changes at 131, 203, and 340 were found to be common among South America strains, as previously reported in studies from Paraguay (Aquino et al., 2008) and Brazil (Oliveira et al., 2010). We propose that amino acids 131 and 340 could be used to place DENV-2 American/Asian into one of two lineages. Notably, residue 131 is located at the interface between domains I and II of the envelope region within a pH-dependent hinge region which becomes folded into domain III during membrane fusion. Mutations in this region may have consequences for conformational changes and the pH threshold during the fusion process (Modis et al., 2004). Lineage II showed a non-conservative substitution at residue 131, from a hydrophobic residue to a hydrophilic polar amide as compared with lineage I.

Taken together, our results suggest that different genetic variants of American/Asian genotype DENV-2 were introduced into Peru from surrounding countries, including Brazil, Bolivia, and Ecuador. It appears that lineage II (clade F) of the American/Asian genotype is a particularly virulent strain due to its emergence in northeastern Peru resulted in the largest DHF epidemic that region had ever experience, even after more than 20 years of DENV circulation (Durand et al., 2011). These findings improve our knowledge of the molecular evolution and emergence of DENV-2 in Peru and highlight the need for continuous monitoring for the emergence or introduction of new DENV genotypes in South America that may be associated with severe disease.

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Copyright statement

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