



## Genetic and phenotypic characterization of sylvatic dengue virus type 4 strains

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### ABSTRACT

Four serotypes of dengue virus (DENV 1–4) currently circulate between humans and domestic/peridomestic *Aedes* mosquitoes, resulting in 100 million infections per year. All four serotypes emerged, independently, from sylvatic progenitors transmitted among non-human primates by arboreal *Aedes* mosquitoes. This study investigated the genetic and phenotypic changes associated with emergence of human DENV-4 from its sylvatic ancestors. Analysis of complete genomes of 3 sylvatic and 4 human strains revealed high conservation of both the 5'- and 3'-untranslated regions but considerable divergence within the open reading frame. Additionally, the two ecotypes did not differ significantly in replication dynamics in cultured human liver (Huh-7), monkey kidney (Vero) or mosquito (C6/36) cells, although significant inter-strain variation within ecotypes was detected. These findings are in partial agreement with previous studies of DENV-2, where human strains produced a larger number of progeny than sylvatic strains in human liver cells but not in monkey or mosquito cells.

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### Introduction

There is growing concern about the potential emergence of new pathogens, particularly arthropod-borne viruses (arboviruses), from animal reservoirs into humans (Weaver and Reisen, 2010; Wilder-Smith and Gubler, 2008). To gain insight into this process of emergence, it is particularly instructive to study viruses that have completed the trajectory from an enzootic into a human reservoir, such as the four serotypes of dengue virus (DENV-1–4, genus *Flavivirus*, family *Flaviviridae*). These viruses originated in a sylvatic cycle between nonhuman primates, and possibly other enzootic hosts, and arboreal *Aedes* (*Ae.*) mosquitoes. Each serotype emerged independently into a human transmission cycle, wherein humans now serve as the exclusive reservoir and amplification hosts for the endemic/epidemic lineages (Vasilakis et al., 2011). In this human cycle, DENV-1, -2, -3, and -4 are transmitted by domestic and peridomestic *Aedes* mosquitoes, primarily *Ae. aegypti aegypti* and *Ae. albopictus* (Halstead, 1997; Halstead et al., 1964; Rosen et al., 1954; Sabin, 1952; Simmons et al., 1931). The human DENV cycle is presently found in nearly all urban and peri-urban environments throughout the tropics and subtropics. In recent decades, DENV transmission among

humans has intensified due to increased travel, uncontrolled urbanization and lack of effective and sustainable vector control programs (Guzman et al., 2010). By current estimates, DENV infects approximately 100 million people each year in over 100 countries.

Unlike the ancestors of many other human viruses, the ancestral sylvatic cycle of DENV remains extant and has been documented in two foci: one in West Africa involving arboreal *Aedes* spp. (e.g. *Ae. furcifer*, *Ae. luteocephalus*) and primates including patas monkeys (*Erythrocebus patas*), African green monkeys (*Chlorocebus sabaeus*), and Guinea baboons (*Papio papio*) (Cordellier et al., 1983; Diallo et al., 2003, 2005; Hervy et al., 1984; Rodhain, 1991; Saluzzo et al., 1986a; Vasilakis et al., 2008c) and the other in peninsular Malaysia involving *Ae. niveus* s. l. and primates including cynomolgus macaques (*Macaca fascicularis*), pig-tailed macaques (*Macaca nemestrina*) and silvered leaf monkeys (*Presbytis cristata*) (Rudnick, 1986). The continued circulation of sylvatic DENV provides an opportunity to perform comparative studies to elucidate the virus attributes that promote arboviral emergence. However these sylvatic viruses also pose a considerable threat, because they may retain the capacity to re-emerge even as efforts to control circulation of human dengue intensify (Vasilakis et al., 2011), in a manner analogous to urban yellow fever.

Although sylvatic and human DENV strains show substantial genetic differences, our previous studies of DENV-2 demonstrated that such differences do not constitute an adaptive barrier to emergence into the human transmission cycle. Specifically sylvatic DENV-2 showed no detectable deficit relative to human DENV-2 in replication kinetics in

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cultured human or *Ae. albopictus* cells (Vasilakis et al., 2008b), in replication in two proxy measures for human infection, monocyte-derived dendritic cells and SCID mice engrafted with human hepatoma cells (Vasilakis et al., 2007b), or in infection of *Ae. aegypti* and *Ae. albopictus* in vivo (Hanley and Vasilakis, unpublished data). Furthermore, some sylvatic *Aedes* species sympatric with sylvatic DENV are capable of transmitting these viruses and may act as bridge vectors when they move between forest and human habitations. For example in West Africa a highly susceptible vector of sylvatic DENV, the forest-dwelling *Ae. furcifer*, (Diallo et al., 2005), disperses into villages (Diallo et al., 2003), while in Southeast Asia, *Ae. albopictus* disperses from the forest into surrounding agricultural settlements (Smith, 1956). Both species bite humans, resulting in potential transmission of sylvatic DENV to humans. Indeed, several reports have now documented spillover of sylvatic DENV, resulting in infection of individual humans or small outbreaks (Cardosa et al., 2009; Carey et al., 1971; Franco et al., 2011; Monlun et al., 1992; Saluzzo et al., 1986a, 1986b; Vasilakis et al., 2008c). Currently it is not possible to distinguish sylvatic and human DENV infections with antibody-based assays, and thus sylvatic DENV infections may frequently be misclassified as human DENV. Nonetheless two recent reports from Southeast Asia (Cardosa et al., 2009) and West Africa (Franco et al., 2011) reveal that sylvatic DENV infections can result in severe disease.

Collectively, these data indicate that DENV has evolved as an ecological generalist capable of utilizing a broad range of *Aedes* vectors and primate hosts (including humans) and suggest that the public health impact of sylvatic dengue spillover may be substantially greater than is currently appreciated. However to date the vast majority of both experimental (Cox et al., 2011; Mota and Rico-Hesse, 2009, 2011; Vasilakis et al., 2007b, 2008a, 2008b, 2009) and phylogenetic (Vasilakis et al., 2007a) studies of sylvatic DENV have focused exclusively on DENV-2, and it is critical to extend these efforts to encompass the full range of genetic and phenotypic diversities within sylvatic DENV. Thus, we investigated the genetic relationships of sylvatic and human DENV-4 using complete genome sequences from each ecotype. Previous studies have generated phylogenies of DENV-4 that include a only a single sylvatic DENV-4 isolate or a single gene from the all 3 known sylvatic DENV-4 isolates (AbuBakar et al., 2002; Wang et al., 2000); in this study we utilized the complete genome sequences of the three sylvatic DENV-4 isolates as well as 59 human DENV-4 sequences from GenBank that represent the complete genotypic diversity known for this serotype. To better link genetic and phenotypic variations, we also measured the replication kinetics of a subset of human versus sylvatic isolates in both mammalian and mosquito cells in culture.

## Results and discussion

### Phylogenetic analyses

Complete genome sequences from 59 human DENV-4 isolates that span the genetic, geographic and temporal ranges of DENV-4 diversity (Chen and Vasilakis, 2011; Holmes and Twiddy, 2003; Vasilakis and Weaver, 2008; Villabona-Arenas and Zanotto, 2011; Weaver and Vasilakis, 2009) as well as the only 3 known sylvatic DENV-4 strains (Rudnick, 1986) were used for phylogenetic analysis. Fig. 1 shows a representative phylogenetic tree derived from Bayesian analysis; several consensus trees obtained based on Maximum-likelihood (ML) and Bayesian analyses exhibited similar topologies.

All 3 sylvatic DENV-4 strains were genetically distinct from and basal to human DENV-4 strains. Among the sylvatic strains, P73-1120 and P75-514 were more closely related to each other than to the P75-215 strain, an observation that reflects their history. Strain P73-1120 was isolated from a sentinel silver leaf monkey (*P. cristata*) in the Gunong Besut forest reserve in Malaysia in 1973 (Table 1). Two years later an aliquot of the original serum sample collected from the

sentinel monkey was used for experimental infections of silver leaf monkeys, which led to the isolation of P75-514 strain (Rudnick, 1986). Our sequences indicated that, during this single passage, several mutations occurred, which were primarily synonymous and resulted in a 0.1% nucleotide divergence from the parent strain P73-1120 (Table 1). Strain P75-215 was isolated in 1975 from a pool of *Ae. niveus s. l.* mosquitoes collected in the forest canopy of the Gunong Besut forest reserve in late 1974 (Rudnick, 1986).

As shown in previous studies (AbuBakar et al., 2002; Bennett et al., 2003; Foster et al., 2003; Klungthong et al., 2004; Vasilakis and Weaver, 2008; Villabona-Arenas and Zanotto, 2011; Weaver and Vasilakis, 2009), human DENV-4 strains clustered into three major genotypes. Genotype I includes strains from the Philippines, Thailand, Vietnam, Myanmar, Malaysia, Sri Lanka, and India (Cecilia et al., 2011; Dash et al., 2011). The India G11337 strain, which was isolated in 1961 and is therefore one of the oldest DENV-4 strains sampled, is genetically distinct and basal to other isolates of this clade. Genotype II is composed of two distinct sub-lineages: IIa, including strains from Malaysia, Thailand and Taiwan and IIb, comprising strains from the Caribbean and the Americas (Bennett et al., 2003; Foster et al., 2003). Genotype III includes Thai strains sampled between 1997 and 2001 that are distinct from the Thai isolates of genotype II (Klungthong et al., 2004).

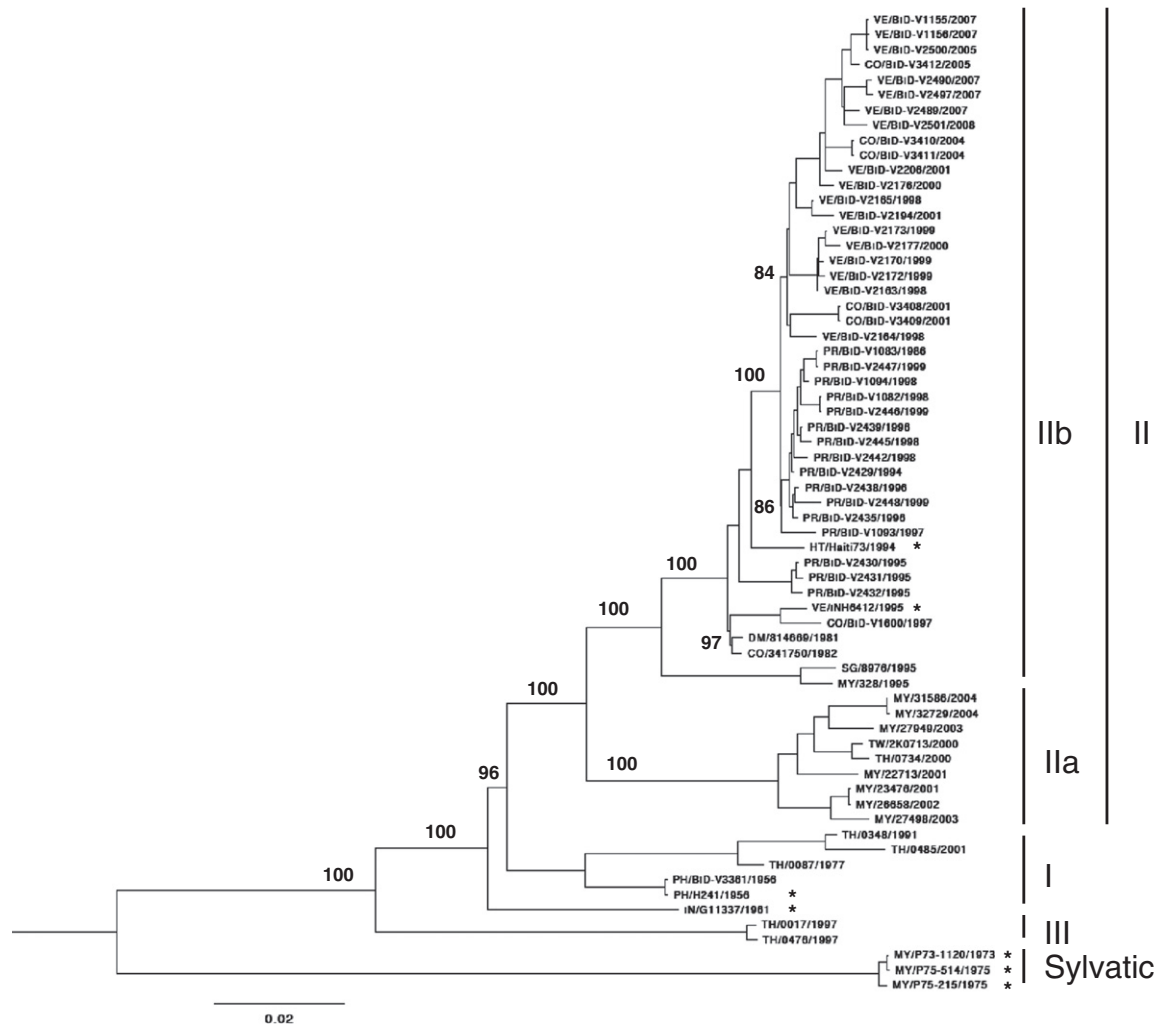
### Genetic analysis

The DENV genome is comprised of approximately 10.7 kilobases (kb) of single stranded RNA of positive polarity. A single open reading frame (ORF) of 10,164 nucleotides (Table 2) is flanked by untranslated regions (UTRs) at the 5' and 3' ends. The 5'-UTR is 101 nt long and is capped with a type I 5'cap (Cleaves and Dubin, 1979; Lindenbach and Rice, 2003), while the 402 nt 3'-UTR lacks the classical polyadenylation site (Wengler et al., 1978) (Table 2).

### 5'- and 3'- UTRs

The sequences of both the 5' and 3' UTRs are highly conserved among the four DENV serotypes (Markoff, 2003). In the DENV-4 strains analyzed, the sequence of both UTRs was highly conserved, and the 5' UTR had a higher sequence identity between the strains (ranging from 95.2 to 98.0%) than the 3' UTR (88.7–95.1%) (Table 2; sequence data is presented in Supplemental Data Figs. 1 and 2). The 5' cyclization sequence (5'CS) located within the coding region of the capsid gene (nt 35–42 after the start codon) (Hahn et al., 1987) was present in all strains analyzed. The flavivirus 3' UTR comprises three sub-regions: the Variable Region (VR), core, and 3' terminus (Markoff, 2003). Several previously identified conserved regions within the core and 3' terminus (RCS2, CS2 and CS1 and CPS (Hahn et al., 1987)) were present in both the sylvatic and human strains (Supplemental Data 3C, D). The highly conserved 3' terminal dinucleotide of the plus-strand (UC-3') was present in all DENV-4 sequences for which the 3' terminal sequence was available, and was complementary to the 5'-terminal dinucleotides (5' AG), purportedly enabling cyclization of the genome during the early stages of replication (Markoff, 2003; Rice et al., 1985; Wengler and Wengler, 1981). Relative to the sylvatic strains, the VR of human genotypes I–III exhibited 3, 13 or 15 nt deletions, respectively (Supplemental Data 2). A similar accrual of deletions in human strains relative to sylvatic strains has been documented in DENV-2 strains (Vasilakis et al., 2008b), and other investigators (Leitmeyer et al., 1999; Shurtleff et al., 2001) have suggested that these deletions occurred during the evolution of human strains from sylvatic progenitors. Proutski et al. (1999) suggested that the VR region may act as a spacer to protect conserved and structurally important distal regions but the precise effects of these particular deletions in human strains have not been determined.

The predicted RNA secondary structures of representative sylvatic (P73-1120) and human (IndiaG11337) DENV-4 strains were extremely similar at the 5' and 3' UTRs (Supplemental Data Figs. 3A–



**Fig. 1.** Phylogenetic analysis of sylvatic DENV-4 isolates. Phylogenetic tree derived from complete genome nucleotide sequences of sylvatic and human DENV-4 strains using Bayesian analysis. Numbers of posterior probability values for monophyletic groups to the right. Asterisks indicate DENV strains used to evaluate replication kinetics. Virus strains are coded by abbreviated country of collection/strain name/year of collection. The scale shows a genetic distance of 0.02 or 2% nucleotide sequence divergence.

D) and were also similar to previously predicted structures for these regions (Markoff, 2003; Rauscher et al., 1997; Romero et al., 2006; Thurner et al., 2004; Yu et al., 2008). As in previous predictions of the 5' UTR in isolation (Brinton and Dispoto, 1988), the RNA secondary structure of each strain contains a long stem loop (SLA) with a shorter side loop (SLB) closest to the start codon; alterations to this sequence may affect RNA-dependent RNA polymerase binding

(Filomatori et al., 2011) and viral replication (Cahour et al., 1995; Filomatori et al., 2006). There has been disagreement in the prediction of the DENV-4 CS2, with some studies showing a dumbbell structure and others showing a single turret (Gritsun and Gould, 2006; Proutski et al., 1999; Romero et al., 2006). The current study found a dumbbell structure at this region for both the sylvatic and human strain. CS2 is particularly important because it is the site of the

**Table 1**

Characterization of genetic sequence of selected dengue-4 sylvatic strains.

Virus <sup>a</sup>	Ecotype <sup>b</sup>	Location	Host <sup>c</sup>	Year	GenBank accession no.	Genome length	% nt identity <sup>d</sup>	% aa identity <sup>d</sup>	ORF (nt)	5'UTR (nt)	3'UTR (nt)
H241	Human	The Philippines	Human	1956	AY947539	10,643 <sup>e</sup>	–	–	10,164	101	282 <sup>e</sup>
IndiaG11337	Human	India	Human	1961	JF262783	10,659	95.1%	99.2%	10,164	101	394
INH6412	Human	Venezuela	Human	1995	JF262781	10,649	94.3%	99.1%	10,164	101	384
Haiti73	Human	Haiti	Human	1994	JF262782	10,649	94.3%	99.1%	10,164	101	384
P73-1120	Sylvatic	Malaysia	<i>A. niveus s.l.</i>	1975	EF457906	10,667	86.9%	98.3%	10,164	101	402
P75-514	Sylvatic	Malaysia	<i>Presbytis cristata</i> (experimental infection)	1975	JF262779	10,667	87.0%	98.3%	10,164	101	402
P75-215	Sylvatic	Malaysia	<i>Presbytis cristata</i> (Sentinel monkey)	1973	JF262780	10,666	86.9%	98.3%	10,164	101	401

<sup>a</sup> Low passage DENV isolates were obtained from the UTMB World Reference Center for Emerging Viruses and Arboviruses and amplified once on C6/36 mosquito cells to achieve sufficiently high titers for further evaluation.

<sup>b</sup> Human indicates isolates associated with the human transmission cycle or associated with peridomestic transmission; sylvatic indicates sentinel monkey or canopy-dwelling mosquito isolate; see also Fig. 1 for phylogenetic groupings.

<sup>c</sup> Source of virus isolation.

<sup>d</sup> Denotes % identity compared to endemic H241 reference strain.

<sup>e</sup> Partial sequence; the 3' terminus of the 3'UTR is missing.

**Table 2**

Nucleotide identity (%) between the consensus sequence of the 5'-UTR (above the diagonal) and 3'-UTR (below the diagonal) of human DENV-4 genotypes I–III and sylvatic DENV-4.

5'-UTR	Genotype I	Genotype II	Genotype III	Sylvatic
Genotype I	100.0	96.2	97.1	98.0
Genotype II	93.1	100.0	95.2	96.2
Genotype III	93.8	95.1	100.0	97.0
Sylvatic	91.9	88.7	88.9	100.0

delta30 mutation that has been used for generation of dengue virus vaccines (Durbin and Whitehead, 2010). Moreover this 30 nt deletion confers attenuation on some but not all DENV strains (Blaney et al., 2004a, 2004b; Durbin et al. 2011). Thus experimental analysis of the structure of this region in sylvatic DENV-4 would be particularly valuable once a reverse genetics system is established.

#### Open reading frame (ORF)

To identify sequence differences between sylvatic DENV-4 and each of the genotypes of human DENV-4, we generated both multiple ORF sequence alignments that included DENV-4 strains from all genotypes as well as human and sylvatic transmission cycles (Fig. 1 and Materials and methods section). Consistent differences between the ancestral sylvatic and derived human strains were inferred to be mutations in the human strains. These comparisons revealed a large number of nucleotide differences, most of which were nonsynonymous (Table 3).

#### Structural proteins

The DENV capsid (C) is a small, positively charged protein (Trent, 1977) containing membrane-associated alpha-helical structures (Jones et al., 2003; Markoff et al., 1997) that form the inner core of the virion. There are 6 positions that show differences between the sylvatic and human strains with most nonsynonymous mutations located outside of predicted alpha-helical regions (Table 3).

The prM protein is the immature precursor to the membrane (M) protein found in the virions, and forms a scaffold over the viral envelope (E) protein to prevent premature fusion during virion maturation (Li et al., 2008). Seven non-synonymous differences between the sylvatic and human DENV4 strains were detected, 2 of which are found within the mature M protein (Table 3).

The E protein comprises the majority of the surface area on the mature virion and is responsible for receptor binding (Chen et al., 1996; Johnson et al., 1994; Rey et al., 1995) and host cell surface fusion (Modis et al., 2004). Of the 29 amino acid changes (Table 3), no changes occurred to the 12 conserved disulfide bridge-forming cysteine residues, to the two N-linked glycosylation sites in the E protein at residues N67 and N153 (Johnson et al., 1994; Zhao et al., 1986), or to proposed heparan sulfate binding sites (Chen et al., 1997; Modis et al., 2005). Given strong antibody neutralization of both sylvatic and human strains of the same serotype by antibodies raised against either ecotype (Vasilakis et al., 2008a) it was not surprising that highly immunogenic residues 104, 106, 107 (located in the fusion loop) and 126, 226 and 231 (Crill and Chang, 2004) remain unchanged between sylvatic and human strains. Interestingly, mutations were observed within the DIII region at positions 310 and 364, which are potentially significant since they are located within the binding domains of several well-characterized monoclonal antibodies including 4E11 (Thullier et al., 2001) and 9D12 (Crill et al., 2009).

#### Nonstructural proteins

The functions of NS1 are not completely known. Like the E protein, NS1 contains 12 disulfide bridge-forming cysteine residues and encodes N-linked glycosylation sites at N130 and N207 (Pryor and Wright, 1994). Of the 26 non-synonymous differences between the sylvatic and human DENV-4 strains, none involved critical cysteines

**Table 3**

Summary of consistent amino acid differences between sylvatic and human DENV-4 genotypes.

Gene	Site <sup>a</sup>	Type <sup>b</sup>	AA substitution	Comment	
C	8	I–III	A → V	T → T: PR 1995 strains	
	34	III	T → I		
	34	II	T → S		
	74	III	N → T		
	81	I–II	T → I		
	102	III	M → V		
	102	I–II	M → I		
	111	I–III	I → V		
	prM	5	I–III		S → T
		41	I–III		V → L
		70	I–III		S → L
76		I–III	T → M		
M (mature)	83	III	S → N	I → I: G11337;0348;0087; 0485; BID-V3361	
	6	I–III	A → T		
E	17	I–III	T → A		
	4	III	V → I		
NS1	19	I–III	T → A	S → S: G11337;0348;0087; 0485; BID-V3361	
	46	I–II	I → T		
	132	I–III	V → I		
	148	I–III	A → T		
	154	I–III	S → D		
	162	I–III	T → A		
	174	III	K → E		
	182	III	T → S		
	202	III	K → E		
	203	I–III	G → K		
	233	III	Y → H		
	265	I – II	T → A		
	310	III	K → R		
	329	I–II	T → A		
	335	I–III	I → V		
	340	I–III	K → R		
	342	I–III	M → V		
	351	I	I → V		
	355	I–III	I → T		
	357	II	F → L		
	364	I – III	I → V		
	382	I–III	A → V		
	384	II	D → N		
	455	III	I → V		
	461	I–II	I → F		
	461	III	I → L		
474	III	S → P			
478	I–III	S → T			
NS1	9	III	S → T	L → L: G11337;0348;0087; 0485; BID-V3361	
	22	I–II	I → V		
	22	III	I → T		
	33	III	K → Q		
	57	I–III	V → I		
	90	I–III	T → A		
	98	III	T → V		
	103	I–III	V → A		
	104	I–III	I → L		
	105	I–III	A → T		
	129	III	R → K		
	131	I–III	N → S		
	152	II	F → S		
	153	I	L → F		
	156	I–III	D → E		
	166	III	N → S		
	171	I–III	L → F		
188	I–III	V → I			
192	I–III	R → K			
246	I–II	T → S			
251	I–III	S → F			
265	II	M → Y			
275	I–III	M → I			
292	I–III	G → D			
338	I–III	S → L			

(continued on next page)

**Table 3** (continued)

Gene	Site <sup>a</sup>	Type <sup>b</sup>	AA substitution	Comment	
NS2A	341	III	K→R		
	4	III	P→S		
	4	I-II	P→T		
	19	III	M→I		
	26	III	R→K		
	38	III	A→T		
	38	II	A→I		
	40	III	L→F		
	54	I-III	V→L		
	57	I-II	I→L		
	65	III	F→L		
	65	I-II	F→S		
	66	III	G→S		
	68	III	M→V		
	68	I-II	M→I	M→M: G11337;0348;0087; 0485; BID-V3361	
	72	III	V→T		
	78	III	A→I		
	113	II	F→L		
	127	I-III	A→S		
	135	I-II	M→I	M→M: G11337;0348;0087; 0485; BID-V3361	
	137	I-III	Y→T		
	138	II	H→Q		
	142	I-III	A→T		
	144	I-III	L→V		
	155	I-III	K→R		
	156	I-III	T→S		
	161	III	I→T		
161	I-II	I→V			
169	I-III	V→A			
173	III	V→A			
197	I-II	I→L			
217	I-II	K→R	K→K: G11337;0348;0087; 0485; BID-V3361		
NS2B	55	III	K→R		
	73	I-III	V→I		
	94	I-III	L→M		
	116	III	V→I		
	119	II-III	A→T		
	127	III	K→R		
NS3	12	I-II	T→A		
	13	I-II	A→T	A→A: G11337;0348;0087; 0485; BID-V3361	
	17	II	M→A		
	17	I, III	M→T		
	19	I-II	T→S	T→T: G11337;0348;0087; 0485; BID-V3361	
	31	I-II	L→F		
	40	I-III	V→I		
	42	I-III	V→M		
	87	I-III	E→D		
	100	I-III	V→I		
	142	II	K→R		
	170	I-III	K→R		
	190	I-III	V→I		
	249	I-III	A→S		
	292	I	S→C	S→S: G11337;0348;0087; 0485; BID-V3361	
	321	II	T→A		
	322	II-III	I→T		
381	I	K→R	K→K: G11337;0348;0087; 0485; BID-V3361		
NS4A	518	III	R→K		
	538	I-III	R→K		
	20	I	R→K	R→R: G11337;0348;0087; 0485; BID-V3361	
	58	I - III	I→V		
	83	III	M→V		
	87	II	A→T		
	97	I-III	I→V		
	140	I-II	A→T		
	141	I-III	L→I		
	146	I-III	S→G		
	148	III	I→V		
	NS4B	11	III	T→A	

**Table 3** (continued)

Gene	Site <sup>a</sup>	Type <sup>b</sup>	AA substitution	Comment
NS5	18	I-III	E→V	
	20	I - III	P→T	
	87	I-III	S→G	
	93	I-III	L→M	
	175	I	A→V	A→A: G11337;0348;0087; 0485; BID-V3361
	187	I	F→L	F→F: G11337;0348;0087; 0485; BID-V3361
	198	I-II	A→I	
	198	III	A→V	
	199	I	L→M	L→L: G11337;0348;0087; 0485; BID-V3361
	205	I - III	G→N	
	240	I-III	T→A	
	42	I-III	L→S	
	45	I	K→R	K→K: G11337;0348;0087; 0485; BID-V3361
	70	II	I→V	I→I: G11337;0348;0087; 0485; BID-V3361
	90	I-III	V→M	
	100	I-III	R→K	
	102	I-III	F→Y	
	163	I-III	I→V	
	165	I-III	R→K	
	199	I	K→R	K→K: G11337;0348;0087; BID-V3361
	200	I-III	Y→H	
	203	I, III	N→S	
	205	I-II	I→V	
	233	I-III	V→T	
	234	I	T→I	T→T: G11337;0348;0087; BID-V3361
	235	I-III	K→R	
	275	I-III	V→I	
284	I	Q→R	Q→Q: 0348;0087;0485; BID-V3361	
320	I-III	I→V		
367	III	P→L		
371	I-II	V→M		
372	I	V→I	V→V: 0348;0087;0485; BID-V3361	
386	I-II	R→K		
419	I-III	A→T		
432	I-III	K→E		
500	I-III	D→E		
520	II	D→E		
523	III	K→R		
525	I-III	E→D		
529	I	M→I		
547	II	L→Q		
559	I-III	G→H		
561	III	K→R		
562	I	I→T	I→I: G11337;0348;0087; 0485; BID-V3361	
585	II	K→R		
642	I-III	K→R		
648	II	K→R		
675	II	S→G		
717	I-III	L→F		
749	I-II	K→R		
785	I-III	V→T		
788	I-III	V→F		
831	I-III	Y→H		
854	I-III	T→S		
864	I-III	Q→H		
885	I-III	A→V		
887	III	K→R		
892	III	P→L		
893	II-III	L→S	L→F: G11337;0348;0087; 0485; BID-V3361; 27949	

**Notes**

Amino acid to the left represents amino acid in sylvatic viruses and to the right on the human viruses. Arrows represent directionality of mutation.

<sup>a</sup> Indicated amino acid location from the start of the gene.

<sup>b</sup> I, II, III: represents genotypes I, II and III of dengue virus serotype 4.

or glycosylation sites. However, one mutation is found in all human genotypes at position 131 (N131S) within the variable residue in the NXT glycosylation signal at this site (Table 3). Furthermore recent evidence suggests that epitope variation within NS1 (Chen et al., 2010; Masrinoul et al., 2011) may influence progression to severe dengue disease by a mechanism of molecular mimicry to human endothelial cell antigens (Liu et al., 2011).

NS2A is a small, hydrophobic protein that in several flaviviruses plays a role in modulating the type I interferon (IFN) response (Munoz-Jordan et al., 2003). Human DENV-4 strains differed from sylvatic strains at 26 amino acids in NS2A (Table 3). The observed high level of variability is characteristic of proteins involved in immune suppression (Franzosa and Xia, 2011; Obbard et al., 2009). NS2B is another small, hydrophobic protein that acts as a cofactor

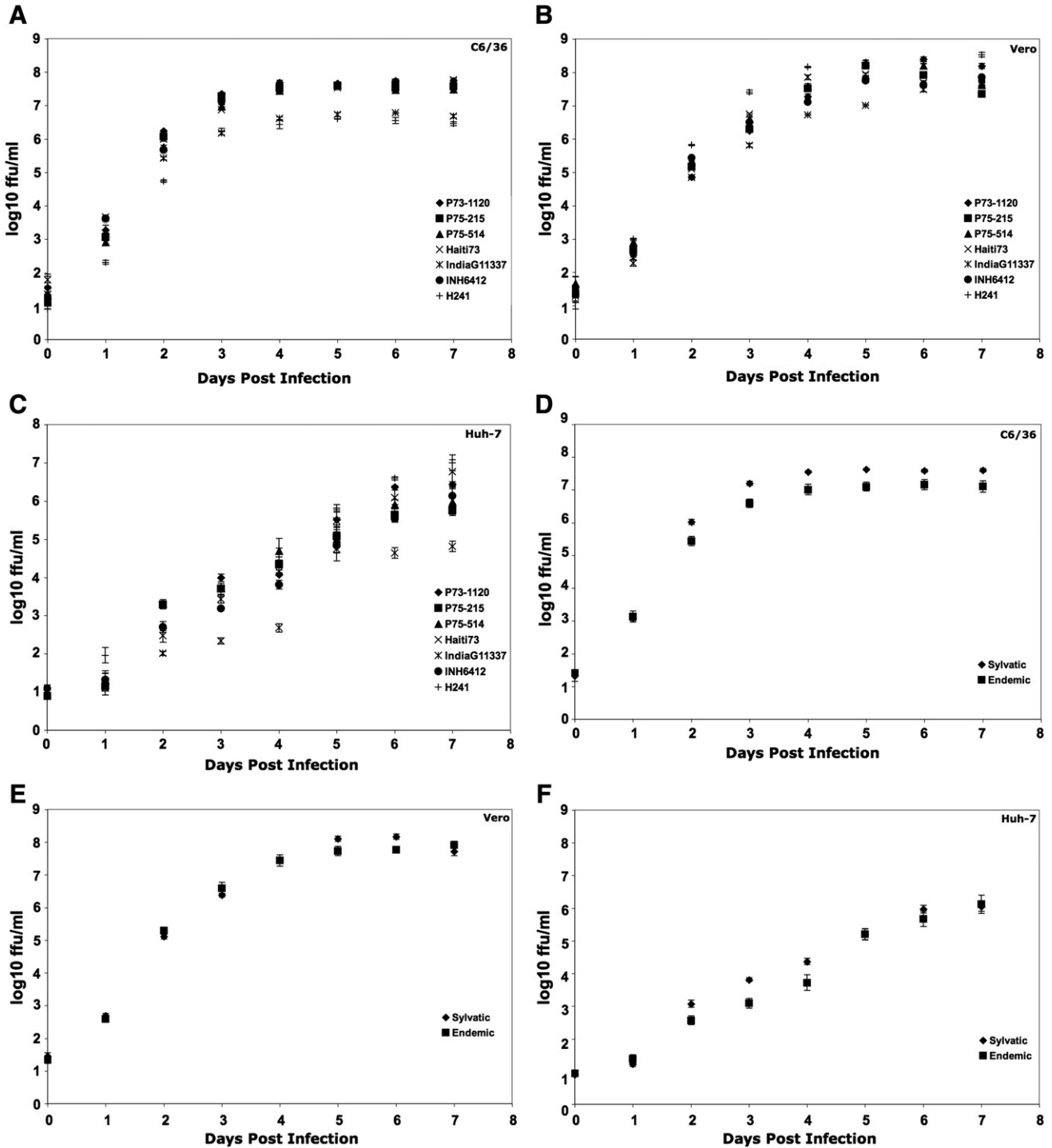


Fig. 2. Comparative replication curves of DENV-4 strains. A–C: Daily virus output from days 0–6 following infection at MOI 0.01 by sylvatic (P73-1120, P75-215 and P75-514) and human (Haiti73, IndiaG11337, INH6412 and H241) DENV-4 strains in mosquito cell line C6/36 (*Ae. albopictus*) and vertebrate cell lines Vero (African green monkey kidney) and Huh-7 (human hepatoma) (A–C, respectively). D–F: Mean virus output of human (squares) and sylvatic (diamonds) DENV-4 strains in C6/36, Vero, and Huh-7 cells (D–F, respectively); means derived from 3 strains of sylvatic and four human DENV-4 shown in panels A–C. The limit of detection of the assay is 0.9  $\log_{10}$  ffu/mL.

for the enzymatic activity of NS3. Six nonsynonymous mutations were detected (Table 3), none of which occurred at critical residues) in NS2B required for NS3 function (Niyomrattanakit et al., 2004). NS3, along with NS2B, has multiple functions including protease and helicase. Nineteen non-synonymous differences were noted (Table 3).

NS4A and NS4B are small, multifunctional, hydrophobic proteins that have recently been shown to play a role in modulating the host's type I IFN responses (Jones et al., 2005; Munoz-Jordan et al., 2003, 2005). Other functions of NS4A have not been fully elucidated, but it associates with host cell membranes (Jiang et al., 2009; Miller et al., 2007) and is required for viral replication (Tajima et al., 2011). Nine differences were observed (Table 3) in human NS4A DENV proteins. NS4B, which has also been shown to act as a helper for the helicase function of NS3 (Umareddy et al., 2006) contained 11 differences between sylvatic and human strains (Table 3). A previous study examining the evolutionary pressures on sylvatic DENV-2 identified 13 sites in the NS4B gene under weak positive selective pressure (Vasilakis et al., 2007a). This observation was striking in that no such sites were identified in human DENV-2, and relatively few positively selected sites were identified in any of the other genes in either of the two ecotypes. Four of the observed NS4B mutations in this current study of DENV-4 (positions 11, 18, 20 and 198) were adjacent to sites under positive selection in sylvatic DENV-2 (Vasilakis et al., 2007a).

NS5 acts as the RNA-dependent RNA-polymerase (RdRp) and is responsible for RNA cap processing. Recently, anti-IFN activity of NS5 has also been reported (Ashour et al., 2009). Forty-eight non-synonymous differences were found in NS5 (Table 3), half of which were seen in all genotypes. The N-terminus region has shown to be responsible for methyltransferase activity, and residues critical for this process (positions 14, 18, 29, 61, 146, 150, 181 and 217) were unchanged between sylvatic and human strains (Egloff et al., 2002). Five non-synonymous mutations occur within the interdomain region between the N-terminal methyl transferase and the C-terminal RdRp (positions 320–405) which contains two nuclear localization signals (NLS) (Pryor et al., 2007), however all critical residues were unaffected.

### Phenotypic analyses

Replication kinetics of four human DENV-4 strains (Genotype I IndiaG11337 and H241, as well as Genotype II Haiti73 and INH6412, Table 1) and the 3 available sylvatic strains (P73-1120, P75-215 and P75-514, Table 1) were characterized in primate kidney and human hepatoma (Vero and Huh-7, respectively) and *Ae. albopictus* mosquito (C6/36) cells. Cultured cells are admittedly an imperfect model of dengue replication in vivo; notably Vero cells and C6/36 cells are deficient in the IFN (Mosca and Pitha, 1986) and RNA interference response (Brackney et al., 2010; Scott et al., 2010), respectively. However replication dynamics in these cells is sometimes correlated with replication in vivo (Hanley et al., 2003) and therefore they offer a useful first screen for phenotypic variation among virus strains.

Titers of most viruses belonging to both human and sylvatic genotypes increased in both C6/36 and Vero cells until day 4 post-infection (p.i.) (Figs. 2A and B respectively), and in Huh-7 cells until day 5 p.i. (Fig. 2C), after which titers stabilized or declined in all cell types. Huh-7 cells exhibited pronounced cytopathic effect (CPE) by day 4 p.i., which became severe by 6 p.i. in response to infection with all DENV-4 strains (data not shown). Vero cells also exhibited CPE following infection by all strains, but it was less severe than that observed in Huh-7 cells; C6/36 cells exhibited no CPE.

Overall, ecotype (human or sylvatic) had no impact on patterns of DENV-4 multistep replication kinetics in any of the 3 cell lines tested ( $P > 0.25$  for all comparisons) (Figs. 2D–F). However significant inter-strain variation was detected within ecotypes during infection of the 3 cell types. The patterns of replication kinetics of individual strains in each cell line are denoted in Figs. 2A–C. In C6/36 cells, both time

( $DF = 7$ ,  $F = 4209.7$ ,  $P < 0.0001$ ) and virus ( $DF = 6$ ,  $F = 132.3$ ,  $P < 0.0001$ ) as well as the interaction between time and virus ( $DF = 42$ ,  $F = 6.5$ ,  $P < 0.0001$ ) had a significant effect on viral replication dynamics. Notably, the human India and H241 strains replicated to significantly lower titers than all other strains (Fig. 2A). In Vero cells, time ( $DF = 7$ ,  $F = 5276.97$ ,  $P < 0.0001$ ) and virus ( $DF = 6$ ,  $F = 115.1$ ,  $P < 0.0001$ ) as well as the interaction between time and virus ( $DF = 42$ ,  $F = 8.7$ ,  $P < 0.0001$ ) had significant effects on replication dynamics. Contrary to its performance in mosquito cells, strain H241 replicated to significantly higher levels than other strains in Vero cells, while the India strain replicated to significantly lower levels than all other strains (Fig. 2B). In Huh-7 cells, both time ( $DF = 7$ ,  $F = 1009.8$ ,  $P < 0.0001$ ) and virus ( $DF = 6$ ,  $F = 29.2$ ,  $P < 0.0001$ ) as well as the interaction between time and virus ( $DF = 42$ ,  $F = 4.6$ ,  $P < 0.0001$ ), had a significant effect on viral replication dynamics. Similar to the pattern observed in Vero cells, H241 replicated to significantly higher levels than most other strains and IndiaG11337 replicated to significantly lower levels than all other strains (Fig. 2C).

In conclusion, our phylogenetic analyses of a diverse array of complete DENV-4 genome sequences demonstrated that sylvatic DENV-4 isolates are basal to and evolutionarily distinct from human DENV-4 isolates, supporting previous classifications of DENV-4 into two discrete ecotypes. This evolutionary and ecologic divergence is further corroborated by our genetic analyses, which revealed considerable differences at both the nucleotide and protein levels between human and sylvatic strains. While all sequence and structural elements previously identified as critical for virus replication (i.e. UTR secondary structures, disulfide bridges, and glycosylation sites) were fully conserved in both ecotypes, nonetheless, we identified several amino acids that may have played a role in the emergence of human DENV-4 strains from sylvatic progenitors. Human and sylvatic DENV-4 strains replicated with similar dynamics in green monkey kidney cells, human hepatoma cells and mosquito cells. These studies in cultured cells should be complemented by testing the phenotypes of sylvatic versus human DENV-4 in peridomestic mosquitoes in vivo and in models for human replication. However the evidence to date suggests that sylvatic strains of DENV-4 in Asia retain the potential to emerge into human transmission cycles.

## Materials and methods

### Viruses and sequencing

To avoid mutations resulting from adaptation to cell cultures, low passage DENV-4 isolates of the following strains were obtained from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at the University of Texas Medical Branch: P75-514, P73-1120 INH6412, JF262781, Haiti73, JF262782, India G11337, and JF262783 (Table 1). All strains were amplified once in cultured C6/36 *Ae. albopictus* cells to achieve sufficiently high titers for amplification and sequencing. We have previously shown that DENV accumulates relatively few mutations during passage in C6/36 cells (Vasilakis et al., 2009). Initial overlapping cDNA fragments and amplicons of viruses were generated using primer pairs specific to DENV P75-215 or H241 in a one-step (Roche Diagnostics, Indianapolis) or two-step (New England Biolabs, MA) reverse transcriptase polymerase chain reactions (RT-PCR) in 8–13 overlapping cDNA fragments spanning the DENV genome. Two-step RT-PCR was generated from a cDNA template using a specific antisense primer (10666: 5'-AGAACCTGTGGATCAACAACACC) and M-MuLV reverse transcriptase (New England Biolabs, Ipswich) as per manufacturer's instructions. The PCR reaction was performed using the high-fidelity Phusion polymerase (Finnzymes, Lafayette, CO) for 35 × amplification cycles at an annealing temperature approximately 5 °C below the lowest T<sub>m</sub>. Amplified sequences were purified (Qiagen, Valencia, CA) and automated sequencing with specific sequencing primers for both strands provided consensus sequences.

### Nucleotide sequence accession numbers

The following DENV-4 genome sequences were determined in this study: INH6412, JF262781; Haiti73, JF262782; India G11337, JF262783; and the sylvatic strains P75-514 and P73-1120, accession numbers JF262779 and JF262780 respectively.

The GenBank accession numbers for the genome sequences of DENV viruses used in the phylogenetic analyses are summarized in Supplemental Data 4.

### Phylogenetic analyses

Sequences were aligned using the ClustalW multiple sequence alignment function of MacVector® version 8.0 (Accelrys, Cary, NC) with default gap penalties. Phylogenetic analyses of the aligned genomic sequences were estimated with maximum likelihood and distance/neighbor joining using the PAUP\* program version 4.10 (D.L. Swofford, Illinois Natural History Survey, Champaign) under the general time-reversible model of nucleotide substitution. The parameter values used for the substitution type, optimal base composition, proportion of invariable sites, as well as the shape parameter of the  $\Gamma$  distribution of rate variation among sites were estimated from the data and are available upon request. We also utilized Bayesian analysis (MrBayes v3.1.0) where 4 MCMC tree searches of 4 million generations each were run simultaneously sampling 1 in 100 trees and computing a 50% majority-rule consensus tree out of the last 9800 sampled trees, where the initial 10% of trees removed as burn in. Bootstrapping obtained by the Bayesian analysis was used to place confidence values on grouping within the consensus tree (Felsenstein, 1985). Character evolution was traced using MacClade 4.08 (Sinauer Associates, Sunderland, MA).

### Viral replication kinetics

Comparative multi-step growth curves of all available sylvatic (P73-1120, P75-215 and P75-514) and selected human (Haiti73, IndiaG11337, INH6412 and H241) DENV-4 strains were generated in triplicate on African green monkey kidney (Vero), human hepatoma (Huh-7) and *Ae. albopictus* (C6/36) cells. Huh-7 cells (clone JTC-39) were obtained from the Japanese Health Sciences Foundation, Osaka. All 3 cell lines were plated in 12-well plates at  $3.6 \times 10^5$ ,  $3.6 \times 10^5$ , and  $7.5 \times 10^5$  cells per well respectively, and infected with a MOI of 0.01 per cell in triplicate. Infected dishes containing Huh-7 and Vero cells were incubated for 1 h with periodic gentle rocking at 37 °C, whereas dishes containing mosquito C6/36 cells were incubated at 28 °C. Viral inocula were removed and cell monolayers were washed thrice with PBS to remove unadsorbed virus. Two ml of complete cell media (MEM supplemented with 5% FBS, 2 mM L-GLUTAMINE, 1% nonessential amino acids (NEAA), and 50 mg/ml penicillin/streptomycin) was then added and dishes were incubated at 28 °C or 37 °C for the mosquito or mammalian cell lines respectively. Virus from individual dishes was harvested daily through day 7 pi, clarified by low speed centrifugation, and assayed in C6/36 cells to determine virus titer by focus forming immunoassay (FFA). Virus yield at each timepoint is recorded as FFU/cell, represented as the ratio of the total amount of virus present in the sample by the number of cells originally infected.

### Focus forming assays and immunostaining

Ten-fold serial dilutions of virus in MEM supplemented with 2% FBS and antibiotics (Invitrogen, Carlsbad, CA), were added in duplicate to confluent C6/36 cell monolayers attached to 24-well Costar® (Corning, NY) plates, and incubated for 1 h with periodic gentle rocking to facilitate virus adsorption at 28 °C. Wells were then overlaid with 1 ml of 0.8% methylcellulose (Sigma-Aldrich, St. Louis)

diluted in warm Optimem (Invitrogen) supplemented with 2% FBS, antibiotics and 1% (w/v) L-glutamine and incubated undisturbed for 4 days at 28 °C. Methylcellulose overlay was aspirated and cell monolayer rinsed once with phosphate buffered saline (PBS), pH 7.4 (Invitrogen) followed by fixation with a mixture of ice-cold acetone and methanol (1:1) solution and allowed to incubate for 30 min at room temperature (RT). Fixation solution was aspirated and plates were allowed to air dry. Plates were washed thrice with PBS supplemented with 3% FBS, followed by hour-long incubation with a dengue-specific hyperimmune mouse ascitic fluid. Plates were washed thrice followed by hour-long incubation with a secondary antibody conjugated to horseradish peroxidase (KPL, Gaithersburg, MD). Detection proceeded with the addition of aminoethylcarbazole (AEC) substrate (ENZO Life sciences, Farmingdale, CT) prepared according to vendor instructions.

### Statistical analyses

To compare multistep replication dynamics, repeated measures ANOVA was first used to compare the replication of each of the seven strains tested, with each of the three cell lines analyzed separately. When a significant effect of virus was detected, the differences among the strains were compared using a Tukey–Kramer post-hoc test. Subsequently, the replication of the two ecotypes (sylvatic and human) was compared in each cell line separately using the mean value for each virus strain at each time point, resulting in  $N = 3$  for the sylvatic ecotype and  $N = 4$  for the human ecotype.

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