

Dengue-1 Virus Clade Replacement in Thailand Associated with Enhanced Mosquito Transmission

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1	Dengue-1 Virus Clade Replacement in Thailand Associated
2	with Enhanced Mosquito Transmission
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5	RUNNING TITLE: Role of vectors in dengue virus evolution
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29 Abstract

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31 Dengue viruses (DENV) are characterized by extensive genetic diversity, which can be 32 organized in multiple, genetically distinct lineages that arise and die out on a regular basis 33 in endemic regions. A fundamental question for understanding DENV evolution is the 34 relative extent to which stochastic processes (genetic drift) and natural selection acting on 35 fitness differences among lineages contribute to lineage diversity and turnover. Here, we 36 used a set of recently collected and archived low-passage DENV-1 isolates from Thailand 37 to examine the role of mosquito vector-virus interactions in DENV evolution. By 38 comparing the ability of 23 viruses isolated on different dates between 1985 and 2009 to 39 be transmitted by a present-day *Aedes aegypti* population from Thailand, we found that a 40 major clade replacement event in the mid-1990s was associated with virus isolates 41 exhibiting increased titers in the vector's hemocoel, which is predicted to result in a 42 higher probability of transmission. This finding is consistent with the hypothesis that 43 selection for enhanced transmission by mosquitoes is a possible mechanism underlying 44 major DENV clade replacement events. There was significant variation in transmission 45 potential among isolates within each clade, indicating that in addition to vector-driven 46 selection other evolutionary forces act to maintain viral genetic diversity. We conclude 47 that occasional adaptive processes involving the mosquito vector can drive major DENV 48 lineage replacement events.

49

50 INTRODUCTION

52	Worldwide, dengue viruses (DENV) are the most important mosquito-borne viral
53	pathogens of humans. The four antigenically distinct DENV serotypes (DENV-1 to -4)
54	cause a broad spectrum of clinical manifestations. An estimated 50 million people
55	experience dengue illness each year, approximately 500,000 of which are associated with
56	severe, life-threatening disease (18). In addition, a significant portion of infections can be
57	inapparent and, thus, go undetected by surveillance programs (15). Despite the large
58	disease burden imposed by dengue on the human population, there is currently no
59	commercially available DENV vaccine or antiviral therapy (46). In endemic regions
60	where multiple serotypes co-circulate, DENV epidemiological dynamics are
61	characterized by complex oscillations in incidence and serotype prevalence (6, 32, 43). A
62	variety of ecological (10, 24) and immunological factors (1, 36) are thought to govern
63	these complex spatio-temporal dynamics. There is also compelling evidence for the
64	influence of virological factors in disease incidence and severity (reviewed in ref. 37).
65	DENV are single-stranded, positive-sense RNA viruses of the genus Flavivirus (family
66	Flaviviridae) with extensive genetic diversity (21). Each serotype can be divided into
67	large, genetically diverse phylogenetic clusters, which, in turn, consist of multiple,
68	distinct lineages (22). Hereafter, we use the terms clade and lineage interchangeably. In
69	the last two decades, in-depth phylogenetic analyses have significantly improved
70	understanding of DENV epidemiological and evolutionary dynamics (reviewed in refs.
71	35, 45).

73 One of the most striking features of DENV evolutionary dynamics is that viral lineages 74 within serotypes arise and die out on a regular basis (22). This lineage turnover is 75 detected in phylogenetic analyses in two different, non-mutually exclusive forms: (i) 76 continuous, "ladder-like" temporal structure within clades and (ii) more dramatic, major 77 clade replacement events. Within a clade, the majority of sub-lineages present at a 78 specific time-point are not detected at later time-points, resulting in a ladder-like tree 79 topology (8, 13, 26). Occasionally, an entire clade that persisted for a number of years at 80 a given location goes extinct as an entirely new clade takes over. Such major clade 81 replacement events have been recurrently documented at regional scales, for example 82 DENV-3 in Sri Lanka in the late 1980s (30), DENV-4 in Puerto Rico during the 1980s-83 1990s (8), DENV-1 in Thailand in the mid-1990s (48), DENV-3 in Thailand in the early 84 1990s (47), DENV-1 in Myanmar in the late 1990s (31), DENV-2 in Vietnam in the early 85 2000s (43), and DENV-1 in Cambodia in the early 2000s (14). Major clade replacement 86 events have also been reported at larger scales. For example, DENV-2 lineages from 87 Southeast Asia displaced a native DENV-2 lineage in the Americas during the early 88 1990s (38). Although successive clades may be temporally non-overlapping (9), in most 89 cases there is a transition period of co-circulation (14, 30, 31, 43, 47, 48).

90

Understanding the causes of DENV lineage birth-and-death has important implications
for dengue epidemiology and control because it is often associated with changes in
disease incidence and severity (16, 17, 30, 31, 38, 40). Elucidating the mechanisms
underlying lineage turnover helps to retrospectively understand spatio-temporal patterns
of dengue incidence and to make predictions about the risk of future epidemics (43). It

also has implications for vaccine design because DENV lineages may differ in their
antigenic properties (39, 44).

98

99 Despite their epidemiological significance, the evolutionary forces underlying major 100 clade replacement events are especially unclear. In particular, the extent to which clade 101 replacement events result from the random sampling of viral variants (genetic drift) due 102 to the stochastic nature of DENV transmission and/or from differences in fitness among variants (i.e., adaptive evolution) is an unresolved question. Analyses of DENV gene 103 104 sequence data have shown that clade replacement events are sometimes associated with 105 signals of positive natural selection, such as an increase in the rate of nonsynonymous 106 substitutions (7), but not always (26, 47). Some phylogenetic studies have concluded that 107 major clade replacements were primarily due to stochastic events (31, 47), whereas others 108 found potentially causal differences in viral fitness between lineages, such as higher 109 viremia level in the human host (43) or enhanced infectivity to mosquito vectors (3, 5, 110 19).

111

Here, we addressed the question whether DENV evolution could be driven by adaptation to mosquito vectors using a set of 23 low-passage DENV-1 isolates from Thailand collected during the period 1985-2009. The 23 isolates were recovered from human serum as part of the routine dengue diagnostic and surveillance services performed at the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok. We compared the ability of the 23 isolates to be transmitted by *Aedes aegypti*, the principal DENV vector species, in experimental *in vivo* vector competence assays. We

119	hypothesized that if viruses are positively selected for enhanced mosquito transmission, a
120	relative increase in transmission potential should be observed for viruses collected over
121	advancing years. All experiments were carried out during 2009-2010 using the first
122	laboratory-reared generation of mosquitoes derived from a wild Ae. aegypti population
123	collected in Kamphaeng Phet, Thailand. Although we cannot be sure that these
124	mosquitoes exactly reproduce the vector-virus interactions that occurred at different dates
125	in the past, they are the most relevant vector population of reference available to test our
126	hypothesis. We estimated virus transmissibility with two distinct, complementary vector
127	competence indices: the proportion of Ae. aegypti females that developed a DENV
128	infection that disseminated from their midgut into their hemocoel, a requirement for virus
129	transmission by a mosquito, and the infectious titer of disseminated virus (27).
130	

131 MATERIALS AND METHODS

132

133 **Mosquitoes.** Wild *Ae. aegypti* immatures (larvae and pupae) were collected from a 134 variety of artificial containers in several households in Nhong Pling, Kon Tee, Nakorn 135 Choom, Na Bo Kham, and Thep Na Korn sub-districts, Muang district, Kamphaeng Phet 136 Province, Thailand during July 2009 (experiment 1) and March 2010 (experiment 2). In a 137 preliminary experiment, immatures were collected from containers in villages of 138 Ladkrabang district, Bangkok, and Muang district, Kamphaeng Phet Province, in January 139 2009. F_0 adults were allowed to emerge in the laboratory, mate randomly, and feed on 140 defibrinated sheep blood (National Laboratory Animal Center, Mahidol University, 141 Bangkok, Thailand) through a membrane feeding system. F_1 eggs, which were collected 142 and stored on dry pieces of paper towel and maintained under high humidity, were 143 hatched synchronously by placing them under low pressure for 30 min. Larvae were 144 reared in 24 x 34 x 9 cm plastic trays filled with 2.0 l of dechlorinated tap water at a 145 density of approximately 200 first instars per tray and fed a standard diet of approximately 1.0 g of fish food pellets (C.P. Hi Pro[®], Perfect Companion Group Co. 146 147 Ltd, Bangkok, Thailand) per tray. After emergence, F_1 adults were housed in plastic 30 x 148 30 x 30 cm cages (Megaview Science Education Service Co. Ltd, Taichung, Taiwan) 149 with permanent access to 10% sucrose. They were maintained under standard insectary 150 conditions at 28±1°C, 80% humidity, and with a 12:12 hour light:dark cycle. 151 152 Virus isolates. Viruses were originally isolated and archived as seed stocks from serum

153 samples collected during routine surveillance for diagnostic public health testing at

154 AFRIMS from clinically ill dengue patients attending Kamphaeng Phet Provincial

155 Hospital in Kamphaeng Phet (experiment 1) and Queen Sirikit National Institute of Child

156 Health in Bangkok (experiment 2). Virus isolation and identification was performed as

157 previously described (25). Each isolate underwent 3-4 passages in cell culture (Table 1),

158 which, according to standard procedures in our AFRIMS laboratory, is the minimum

required to obtain a viral titer sufficiently high to infect mosquitoes orally using anartificial blood meal.

161

162 **Oral challenge.** Two sets of two-day-old confluent cultures of *Aedes albopictus* cells (C6/36, ATCC #CRL-1660TM) in 25 cm² flasks (approximately 10⁷ cells/flask) were 163 164 inoculated with 1.0 ml of stock virus per flask and incubated at 35°C under 5% CO₂. A 165 similar titer can be reached by growing virus in C6/36 cells at 35°C for 4-6 days instead 166 of the usual 7-10 days at 28°C. In experiment 1, supernatant was harvested at 5 and 6 167 days post-inoculation to prepare the infectious blood meal of experimental blocks 1 and 168 2, respectively. In experiment 2, supernatant was harvested at 4 days post-inoculation to 169 prepare the infectious blood meal of both experimental blocks. The protocol difference 170 between experiments 1 and 2 was due to logistical constraints. The artificial blood meal 171 consisted of 1:1 mix of defibrinated sheep blood (National Laboratory Animal Center, 172 Mahidol University, Bangkok, Thailand) and virus suspension. Three- to seven-day-old 173 Ae. aegypti F_1 females deprived of sucrose and water for 24 hours were offered an 174 infectious blood meal for 30 min through pieces of desalted porcine intestine stretched 175 over water-jacketed glass feeders maintained at 37°C. Samples of the blood meal were saved for subsequent titration by plaque assay. After blood feeding, mosquitoes were 176

briefly sedated with CO₂ from dry ice and fully engorged females were transferred to
clean paper cups. Unfed or partially fed females were discarded. Engorged females were
maintained under standard insectary conditions and provided cotton soaked with 10%
sucrose *ad libitum*.

181

182 Vector competence. The ability of DENV isolates to be transmitted by *Ae. aegypti* was 183 assessed at 7 and 14 days post blood meal (pbm) with two vector competence phenotypes: (i) the proportion of mosquitoes that developed a disseminated infection and 184 185 (ii) the infectious titer of disseminated virus. These two complementary indices of vector 186 competence represent two successive, non-overlapping aspects of the infection process in 187 mosquitoes that lead to their ability to transmit virus, namely that virus disseminates 188 beyond the midgut and the magnitude of infectious titer in the hemocoel. As such, we 189 analyzed them separately. The two periods of extrinsic incubation (7 and 14 days pbm) 190 were chosen to represent early and late phases of dissemination kinetics (28). 191 Relationship between the infectious titer of disseminated virus and the potential for 192 transmission *in vitro* was established in a preliminary experiment (see below). Upon 193 harvest, the head of each female was cut off and placed individually in 1.0 ml of 194 mosquito diluent (MD), consisting of RPMI 1640 medium with 10% heat-inactivated 195 FBS with 100 units/ml penicillin and 100 µg/ml streptomycin. Bodies were kept 196 separately in 1.0 ml of MD. Samples were stored at -70° C before processing. Body and 197 head samples were quickly thawed in a water bath at 35±2°C and homogenized in a 198 mixer mill (Qiagen) at 24 cycles/sec for 2 min. Infected bodies were screened by 199 serotype-specific RT-PCR (experiment 1) or plaque assay (experiment 2). In experiment

200	1, total RNA was extracted from 140 µl of body homogenates using QIAamp viral RNA
201	mini kit (Qiagen) according to the manufacturer's instructions. RT-PCR was performed
202	with 5 μ l of extracted RNA following a standard protocol (29) with the following
203	modifications: (i) 1X PCR buffer II supplied with Amplitaq DNA Polymerase (Applied
204	Biosystems) was used instead of the original buffer (50 mM KCl, 10 mM Tris pH 8.5 and
205	0.01 mM gelatin) in both the first round RT-PCR and the second round PCR (nested
206	PCR); (ii) the first round RT-PCR reaction contained Avian myeloblastosis virus reverse
207	transcriptase (Promega) instead of rav-2 recombinant reverse transcriptase; (iii) the 1:50
208	dilution of the first round RT-PCR product was used as the template in the nested PCR;
209	(iv) the nested PCR reaction contained 12.5 pmol of each primer instead of 50 pmol; and
210	(v) the number of the nested PCR cycles was increased from 20 cycles to 25 cycles (29).
211	Plaque assay was performed in rhesus monkey kidney cells (LLC-MK ₂ , ATCC #CCL-
212	7^{TM}) as described previously (42). Briefly, the homogenized samples were passed
213	individually through a 0.22 μ m syringe filter unit and 1:2, 1:10 and 1:100 dilutions were
214	prepared in MD. The samples were placed in an ice bath and 100 μ l/well were inoculated
215	into a monolayer of LLC-MK $_2$ cells in 24-well plates. The virus was adsorbed for 1 hour
216	at room temperature (20-28°C) on a rocker platform. The inoculum was removed and 0.5
217	ml/well of a first overlay of medium was added. The cells were incubated for 5 days at
218	$35\pm1^{\circ}$ C in a $5\pm0.5\%$ CO ₂ incubator. The cells were stained with a second overlay of
219	medium containing 4% neutral red (Sigma). Plaques were counted and plaque forming
220	units (PFU)/ml were calculated. Head samples of infected bodies were titrated by plaque
221	assay in LLC-MK ₂ cells. Mosquitoes whose bodies were negative by RT-PCR

(experiment 1) or plaque assay (experiment 2) were considered uninfected and theirheads were not processed further.

225	In vitro transmission. A preliminary experiment was carried out to define the
226	relationship between the titer of disseminated virus and the potential for in vitro virus
227	transmission. This experiment involved two F ₁ populations of Ae. aegypti sampled during
228	January-February 2009 in Bangkok and Kamphaeng Phet, respectively. Both populations
229	were experimentally exposed to two 2009 DENV-1 isolates from Bangkok (ID#
230	00076/09 and 00088/09) and two 2009 DENV-1 isolates from Kamphaeng Phet (ID# \ensuremath{O}
231	30015/09 and 30025/09). The relationship between the titer of disseminated virus and <i>in</i>
232	vitro transmission success was evaluated both at 8 and 14 days pbm. Mosquitoes were
233	anesthetized with triethylamine (Sigma-Aldrich), and their legs were removed
234	individually and placed into 1.0 ml of MD. Saliva samples were collected using a forced
235	salivation technique (2). Briefly, mosquito mouthparts were inserted for 15 min into a
236	glass microcapillary tube filled with approximately 10 μ l of FBS. After 15 min, the
237	contents of the microcapillary tube were placed into 0.3 ml of MD and snap-frozen in dry
238	ice. The remainder of the mosquito bodies were placed individually into 1.0 ml of MD,
239	and all samples were stored at -70° C before processing. Body and leg samples were
240	quickly thawed in a water bath at 35±2°C and homogenized in a mixer mill. Infected
241	bodies were screened by serotype-specific RT-PCR as described above. The titer of
242	disseminated virus in the legs of mosquitoes whose body was positive by RT-PCR was
243	determined by plaque assay in LLC-MK ₂ cells as described above. Mosquitoes whose
244	bodies were negative by RT-PCR were considered uninfected and their legs were not

processed further. The presence of virus in the saliva samples from mosquitoes with a
disseminated infection was tested by plaque assay in LLC-MK₂ cells.

247

248 Sequencing and phylogenetic analysis. Viral RNA extracted by QIA amp viral RNA 249 mini kit was converted to cDNA using Transcriptor High Fidelity kit (Roche) and random 250 hexamer oligonucleotides according to the manufacturer's instructions. Sequencing of E 251 genes and complete genomes was performed following previously described methods 252 (48). The DNA fragments of the E gene and the overlapping DNA fragments covering the 253 entire DENV-1 genome were amplified using Amplitaq DNA Polymerase (Applied 254 Biosystems) and purified using QIAquick PCR purification kit and QIAquick gel 255 extraction kit (Qiagen). Purified DNA fragments were used as templates in cycle 256 sequencing reactions using the DYEnamic ET dye terminator sequencing kit (GE 257 Healthcare) according to the manufacturer's instructions. The sequencing primers were 258 described previously (48). The sequencing products were purified by standard ethanol 259 precipitation before sequencing in a MegaBACE 500 automated DNA sequencer (GE 260 Healthcare). The overlapping sequences obtained from forward and reverse primers were 261 combined for analysis and edited by using the Sequencher software (Gene Code 262 Corporation). Phylogenetic trees were constructed with PAUP* version 4.0 (41) using a 263 maximum likelihood (ML) method. ML trees included the new sequences generated in 264 this study and 17 "background" DENV-1 sequences from GenBank. The best model of 265 nucleotide substitution was chosen with jModelTest (33) based on the lowest akaike 266 information criterion (AIC) value (34). The TIM2+ Γ and GTR+ Γ models of nucleotide 267 substitution were applied to construct the trees for E gene and complete genome

sequences, respectively. Reliability of particular phylogenetic groupings was calculated
using bootstrap resampling analysis with 1,000 replicate neighbor-joining (NJ) trees
under the ML substitution models. All sequences have been submitted to GenBank and
assigned accession numbers JN638322-JN638344.

272

273 Data analysis. All statistical analyses of vector competence data included the 274 confounding effects of the experiment and the experimental block. The study was run in 275 two separate experiments that involved two different sets of DENV isolates and used a 276 population of *Ae. aegypti* that was sampled at two different times. In both experiments, 277 the same batch of mosquitoes was exposed twice to the same set of DENV isolates on 278 two successive days (i.e., two experimental blocks). In the two blocks, viruses came from 279 the same passage in cell culture but were harvested separately (see above). The effect of 280 the block was thus nested within each experiment and accounted for the difference in titer 281 between the two harvests, the effect of the one-day difference in mosquito age, and the 282 effect of any uncontrolled differences between the two days. The isolate effect was nested 283 within each experiment and clade because each isolate belonged to only one phylogenetic 284 clade and each experiment involved a different set of isolates. Blood meal titers were log-285 transformed (to ensure normal distribution of the residuals) and compared using a 286 multifactorial analysis of variance (MANOVA) accounting for the effects of clade, 287 experiment, block and their interactions. The proportion of mosquitoes with a 288 disseminated infection was analyzed with a nominal logistic regression that included the 289 effects of blood meal titer, experiment, experimental block, clade, isolate and their interactions. The titer of disseminated virus was log-transformed (to ensure normal 290

- distribution of the residuals) and analyzed with a MANOVA that included the effects of
- blood meal titer, experiment, experimental block, clade, isolate and their interactions.
- 293 Differences were considered statistically significant at P<0.05. Analyses were performed
- with the software JMP version 5.1.2.
- 295

296 **RESULTS**

297

317

298 **Estimation of transmission potential.** Transmission potential was defined as the 299 presence of infectious virus in the mosquito's saliva. In a preliminary experiment, a total 300 of 322 individual Ae. aegypti females that developed a disseminated infection following 301 oral challenge were tested for virus titer in their legs and virus presence in their saliva 302 collected *in vitro*. The data included two F₁ Ae. aegypti populations (sampled in Bangkok 303 and Kamphaeng Phet, respectively), four DENV-1 isolates (two from Bangkok and two 304 from Kamphaeng Phet), and two extrinsic incubation periods (8 and 14 days pbm). Leg titers ranged from 1 x 10^1 to 2.3 x 10^4 plaque forming units per ml (PFU/ml) and 305 306 infectious virus was detected in 7.5% of saliva samples. The probability of detecting 307 infectious virus in saliva was positively correlated with the infectious titer of 308 disseminated virus (Fig. 1). Logistic regression showed that the leg titer was a highly significant predictor of virus detection in saliva (df=1; L-R χ^2 =15.48; P<0.0001). In 309 310 particular, no virus was detected in any of the saliva samples from mosquitoes with a leg titer $< 1 \times 10^3$ PFU/ml. For the rest of the study, the infectious titer of disseminated virus 311 312 was used as an estimate of transmission potential. Because the number of mosquito legs 313 may vary among individuals (they can lose them over time), the head titer was used 314 instead of the leg titer. Previous data showed that the leg titer and the head titer are 315 strongly correlated in individual Ae. aegypti (27). 316

318 isolates were passaged four times in cell culture (Table 1) prior to their use in

16

Phylogenetic relationships between DENV isolates. All but two of the 23 DENV-1

319 experimental mosquito infections; two were passaged three times. The study was run in 320 two separate experiments involving two different sets of virus isolates (11 in the first experiment and 12 in the second) and Ae. aegypti mosquitoes sampled at two different 321 322 times. Phylogenetic analysis based on the complete DENV E gene sequence indicated 323 that the virus isolates could be divided into two major phylogenetic clades within the 324 genotype I of DENV-1 (Fig. 2A). The first clade included 8 isolates sampled between 325 1985 and 1995; the second clade included 15 isolates sampled between 1992 and 2009. 326 Hereafter, the first and second clades are termed "early clade" and "new clade", 327 respectively, in reference to the chronological order of their detection. Complete genome 328 sequences obtained for a selection of 9 isolates sampled between 1986 and 1997 (6 from 329 the early clade and 3 from the new clade) confirmed the phylogenetic relationships 330 inferred using the *E* gene sequence (Fig. 2B).

331

332 Variation in transmission potential among DENV isolates. Vector competence assays 333 included a total of 738 individual Ae. aegypti females. Transmission potential was 334 characterized based on 7-44 (mean 32) individuals per isolate. Infectious titer of artificial 335 blood meals used in vector competence assays varied among isolates (Fig. 3), ranging from $3.5 \ge 10^4$ PFU/ml to $3.0 \ge 10^6$ PFU/ml. Blood meal titers were significantly higher 336 337 in the second experiment (SS=3.251; df=1; F=19.63; P<0.0001), but they were not significantly different between blocks within experiments (SS=0.574; df=2; F=1.734; 338 339 P=0.191). The higher blood meal titers in experiment 2 may have resulted from the 340 different harvest date post-inoculation in cell culture (day 4) compared to experiment 1 341 (day 5-6). Importantly, blood meal titers did not differ between clades (SS=0.439; df=1;

F=2.649; P=0.112) (Fig. 2). In the early clade, blood meal titers ranged from 4.5 x 10⁴PFU/ml to 3.0 x 10⁶ PFU/ml (mean 7.0 x 10⁵ PFU/ml). In the new clade, blood meal titers ranged from 3.5 x 10⁴ PFU/ml and 2.4 x 10⁶ PFU/ml (mean 8.2 x 10⁵ PFU/ml).

346 Overall, about two thirds of mosquitoes were infected (65.6% at 7 days pbm and 69.6% 347 at 14 days pbm). Nearly all infected females developed disseminated infections within the 348 first two weeks pbm (52.9% at 7 days pbm and 94.6% at 14 days pbm). The percentage 349 of females with a disseminated infection (including uninfected individuals) varied 350 significantly among isolates at both 7 and 14 days pbm (Table 2). It ranged from 0 to 351 81% at 7 days pbm (mean 32%) and from 20 to 100% (mean 64%) at 14 days pbm. The 352 percentage of females with a disseminated infection was not significantly influenced by 353 the blood meal titer, but it was significantly higher in the first than in the second 354 experiment at 14 days pbm (Table 2). The higher level of dissemination in the second 355 experiment may have been due to either the different geographical origin of viruses 356 (Bangkok vs. Kamphaeng Phet) or any uncontrolled, environmental difference between 357 the two experiments. There was no significant difference between the early and new 358 clade in the mean percentage of females with a disseminated infection at either 7 or 14 359 days pbm (Table 2). Dissemination tended to increase as a function of the year of virus 360 isolation in the early clade whereas it tended to decrease as a function of the year of virus 361 isolation in the new clade, both at 7 days pbm (Fig. 4A) and 14 days pbm (Fig. 4B). 362 These trends may have been a consequence of variation in blood meal titers, which 363 showed similar patterns (Fig. 3).

364

365	Among females with a disseminated infection, head titers ranged from 1.0×10^1 to 2.3×1
366	10^4 PFU/ml at 7 days pbm (N=128, mean 5.4 x 10^2 PFU/ml, median 3.0 x 10^1 PFU/ml),
367	and from 1.0 x 10^1 to 2.9 x 10^4 PFU/ml at 14 days pbm (N=243, mean 3.4 x 10^3 PFU/ml,
368	median 1.5 x 10^3 PFU/ml). The titer of disseminated virus was not significantly
369	influenced by the blood meal titer; it differed significantly among isolates at 14 days pbm
370	(Table 3). The mean head titer of the new clade was significantly higher than that of the
371	early clade at 14 days pbm (Table 3; Fig. 5). At 7 days pbm, the new clade had a higher
372	mean head titer than the early clade, but the difference was marginally insignificant
373	(Table 3; Fig. 5). The statistical power at 7 days pbm, however, was reduced compared to
374	14 days pbm because the number of mosquitoes with a disseminated infection was half as
375	large. At 7 days pbm the head titer tended to increase slightly as a function of the year of
376	virus isolation in the new clade, whereas it did not vary temporally in the early clade (Fig.
377	4C). At 14 days pbm, the head titer tended to increase as a function of the year of virus
378	isolation in the early clade, whereas it did not vary temporally in the new clade (Fig. 4D).
379	

DISCUSSION

384	By comparing the ability of 23 DENV-1 isolates from Thailand spanning a 24-year
385	period to infect and be transmitted by Ae. aegypti, we found that a major clade
386	replacement event in the mid-1990s was associated with a higher transmission potential
387	of the isolates belonging to the new clade. Higher transmissibility was mainly due to a
388	higher infectious titer of virus in the vector's hemocoel, which is predicted to result in a
389	higher probability of transmission. This finding supports the hypothesis that major clade
390	replacement events can be driven by natural selection and emphasizes the potentially
391	important role of vector-virus interactions in DENV evolution.
392	
393	The major clade replacement event revealed by the phylogenetic analysis of the 23
394	isolates of our study was described previously (48). In their study, Zhang et al. (48)
395	observed that the DENV-1 clade replacement was associated with a decline in DENV-1
396	prevalence and a concomitant rise of DENV-4 in Thailand. They speculated that clade
397	replacement events might result from differential susceptibility to cross-reactive immune
398	responses in the human host. Here, we provide a non-mutually exclusive, alternative
399	explanation. Our data suggest that this clade replacement event may have been driven, at
400	least in part, by adaptation to mosquito vectors. Even though it occurred in a context of
401	declining DENV-1 incidence, the new clade may have outcompeted the early clade
402	through enhanced mosquito transmission. Isolates in the new clade had a higher
403	dissemination titer, which is expected to result in higher viral fitness because the

404 probability of transmission by a mosquito is positively correlated with the quantity of405 virus that circulates in the insect's hemocoel.

406

407 Our estimate of DENV transmission potential relied on an experiment demonstrating a 408 positive correlation between the titer of disseminated virus and the probability of virus 409 detection in the mosquito's saliva (Fig. 1). Virus presence in the saliva was determined 410 using an established *in vitro* method (2) that likely underestimates transmission 411 probability because it may fail to detect small, but transmissible amounts of virus. Indeed, 412 infectious virus was only detected in the saliva of 7.5% of females with a disseminated 413 infection. Our analysis showed, however, that the probability to detect infectious virus in 414 saliva samples was positively correlated with the infectious titer of disseminated virus, 415 across two different *Ae. aegypti* populations and four different DENV-1 isolates. In 416 particular, there was a lower threshold titer below which none of the mosquitoes with a 417 disseminated infection had a positive saliva sample. Although the actual threshold may be 418 lower than we estimate due to the relatively low sensitivity of the method, our data 419 established that the titer of disseminated virus is a strong predictor of transmission 420 probability in this system.

421

It is worth noting that we did not consider other entomological parameters underlying DENV transmission such as vector survival or biting rate, which could potentially vary among viruses. Future studies will determine whether variation of these traits contribute to fitness differences among viruses. Another limitation of the study was that adaptation to cell culture could have occurred during the 3-4 passages of virus amplification prior to

their use in experimental infections of mosquitoes. The selection regime, however, was
the same for all isolates, thus comparisons among vector competence phenotypes
remained meaningful. The mechanisms underlying the phenotypic differences we
observed between the two clades remain unknown. Authors of a recent study suggested
that variation in vector competence among DENV-2 lineages does not depend on
mosquito midgut binding affinity, but rather on replication ability (12).

434 Investigators who preceded us and studied DENV-2 and DENV-3 indicated that vector-435 driven selection could play an important role in DENV evolution. By comparing the 436 ability of three DENV-3 isolates of a native lineage and three DENV-3 isolates of an 437 invasive lineage to be transmitted by Ae. aegypti, Hanley et al. (19) showed that a clade 438 replacement event in Sri Lanka during the 1980s was associated with enhanced mosquito 439 transmission potential for the invasive lineage. Enhanced transmissibility was associated 440 with a higher body titer and an increased proportion of *Ae. aegypti* females with a 441 disseminated infection (19). At a larger geographic scale, Rico-Hesse and others 442 suggested in a series of studies that displacement of a native DENV-2 lineage in the 443 Americas by Southeast Asian DENV-2 lineages during the early 1990s was driven in part 444 by adaptation to mosquitoes (3-5, 11). In both the DENV-3 and DENV-2 cases, invasive 445 lineages were associated with more severe disease in humans, which highlights the 446 critical need to more fully explore the connection between DENV evolutionary biology 447 and dengue epidemiology.

448

449 Our study helps to understand the respective contributions of stochastic and adaptive 450 processes in DENV evolution. Frequent introductions of new lineages (gene flow) are 451 known to maintain substantial DENV genetic diversity even at a very local scale (23). In 452 contrast, elimination of deleterious mutations (purifying selection) strongly limits *in situ* 453 evolution of lineages (23). Purifying selection is likely the dominant evolutionary force in 454 DENV evolution (20), and nucleotide fixation events are due primarily to genetic drift 455 (13, 20, 26). Although stochastic processes are undoubtedly important in DENV 456 evolution, the present study indicates that more dramatic major clade replacement events 457 may have an adaptive basis (43). That extinction of the early DENV-1 clade was 458 concomitant with rise of the new clade, with an overlapping period in the early 1990s 459 (48), was suggestive of a fitness difference between the two clades. The relatively modest 460 average fitness difference between the two clades (a 0.5-log difference in head titer) may 461 explain why they co-circulated for at least four years before the early clade went extinct. 462 It is worth noting that we also observed significant variation in vector competence indices 463 within each clade (Fig. 4), indicating that other evolutionary forces than vector-driven 464 selection promote DENV genetic diversity. In particular, the temporal pattern within each 465 clade did not provide a clear indication that adaptation to mosquitoes occurs continuously 466 through time (Fig. 4). We speculate that the temporal trends observed within clades 467 resulted from uncontrolled variation in experimental blood meal titers (Fig. 3), but this 468 merits further investigation.

469

470 Although other selective forces may be important drivers of DENV evolution, especially471 those occurring in the human host that we did not examine, we provide evidence for the

- 472 possible role of adaptation to mosquito vectors in a major DENV-1 clade replacement
- 473 event in Thailand in the early 1990s. Our results reinforce the idea that vector-virus
- 474 interactions can play a critical role in DENV epidemiological and evolutionary dynamics,
- 475 and suggest that the role of vector-mediated selection be investigated in other instances of
- 476 clade replacement.

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479

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499 mosquitoes and vector competence assays. R.G.J. coordinated the collection of virus

500 isolates, virological assays, and phylogenetic analyses. T.W.S. conceived and coordinated

- 501 the study, and helped to design the research and write the manuscript. All authors read
- 502 and approved the final manuscript.

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665 **Table 1. DENV-1 isolates used in experimental mosquito infections.** For each isolate,

the year of virus isolation, location of origin, number of passages in cell culture, and

667 phylogenetic group (clade) are indicated. KPPH: Kamphaeng Phet Provincial Hospital;

668 QSNICH: Queen Sirikit National Institute for Child Health (Bangkok); TS:

- 669 *Toxorhynchites splendens* mosquitoes; C6/36: *Aedes albopictus* cells.
- 670

Experiment	Isolation year	Isolate ID	Source	Passage History	Clade
1	1986	KD86-035	KPPH	TS-1, C6/36-3	Early
	1990	KD90-157	KPPH	TS-1, C6/36-2	Early
	1992	KD92-080	KPPH	TS-1, C6/36-2	Early
	1995	30399/95	КРРН	C6/36-4	New
	1997	30231/97	КРРН	C6/36-4	New
	2000	30529/00	КРРН	C6/36-4	New
	2003	30247/03	КРРН	C6/36-4	New
	2005	30230/05	КРРН	C6/36-4	New
	2006	30118/06	КРРН	C6/36-4	New
	2009	30015/09	КРРН	C6/36-4	New
	2009	30025/09	КРРН	C6/36-4	New
2	1985	D85-372	QSNICH	TS-1, C6/36-3	Early
	1986	D86-412	QSNICH	TS-1, C6/36-3	Early
	1987	D87-116	QSNICH	TS-1, C6/36-3	Early
	1990	D90-1197	QSNICH	TS-1, C6/36-3	Early
	1992	03881/92	QSNICH	TS-1, C6/36-3	New

1995	00407/95	QSNICH	TS-1, C6/36-3	Early
1997	00616/97	QSNICH	TS-1, C6/36-3	New
2000	04805/00	QSNICH	TS-1, C6/36-3	New
2003	01417/03	QSNICH	TS-1, C6/36-3	New
2005	00442/05	QSNICH	TS-1, C6/36-3	New
2007	02128/07	QSNICH	C6/36-4	New
2009	00132/09	QSNICH	C6/36-4	New

673 Table 2. Logistic regression analysis of dissemination prevalence 7 and 14 days post-

674 blood meal among mosquitoes experimentally exposed to different DENV-1 isolates.

- 675 df: degrees of freedom; L-R: likelihood ratio. *P < 0.05; **P < 0.01.
- 676

		Dissemination 7		Dissemination 14	
		days pbm		days pbm	
Source	df	L-R χ^2	<i>P</i> -value	L-R χ^2	<i>P</i> -value
Blood meal titer	1	1.451	0.228	0.749	0.387
Experiment	1	1.058	0.304	11.45	0.001**
Clade	1	0.877	0.349	3.619	0.057
Experiment*Clade	1	0.443	0.506	0.461	0.497
Block [within Experiment]	2	1.604	0.448	11.34	0.003**
Block*Clade [within Experiment]	2	0.080	0.961	4.236	0.120
Isolate [within Experiment,	19	34.82	0.015*	46.21	0.001**
Clade]					

677

679 Table 3. Analysis of variance of head titers at 7 and 14 days post-blood meal among

680 mosquitoes experimentally exposed to different DENV-1 isolates. Titers were log-

transformed to satisfy statistical assumptions. The analysis is performed on individual

682 females with a disseminated infection (i.e., excluding those that did not develop a

683 disseminated infection). df: degrees of freedom; SS: sum of squares. **P*<0.05; ***P*<0.01.

684

	Не	ad titer	7 days	pbm	Head titer 14 days pbm			titer 14 days pbm		
Source	df	SS	F	<i>P</i> -	df	SS	F	<i>P</i> -value		
			ratio	value			ratio			
Blood meal titer	1	2.005	4.086	0.046*	1	0.027	0.048	0.828		
Experiment	1	2.651	5.403	0.022*	1	0.353	0.633	0.427		
Clade	1	1.898	3.869	0.052	1	5.494	9.838	0.002**		
Experiment*Clade	1	0.235	0.479	0.491	1	0.007	0.013	0.908		
Block [within	2	0.787	0.802	0.451	2	0.483	0.433	0.649		
Experiment]										
Block*Clade [within	2	3.215	3.276	0.042*	2	3.153	2.823	0.062		
Experiment]										
Isolate [within	19	15.60	1.673	0.054	19	26.76	2.522	0.001**		
Experiment, Clade]										
Error	100	49.06			215	120.1				

685

687 FIGURE LEGENDS

688

689 Figure 1. Mosquito transmission potential strongly correlates with infectious titer of 690 **disseminated DENV-1**. The percentage of female Ae. aegypti tested positive for 691 infectious virus in saliva samples collected *in vitro* is shown as a function of infectious 692 virus titers in their legs at either 8 or 14 days post-blood meal. Bars represent individuals 693 with a leg liter > the lower value of the corresponding interval on the x-axis. Data shown 694 is pooled from vector competence assays involving two mosquito populations exposed to 695 four different DENV-1 isolates (see Materials and Methods for details). Dotted, vertical 696 lines indicate the 95% confidence intervals of percentages. Sample sizes are indicated 697 above the bars. 698 699 Figure 2. Phylogenetic relationships among DENV-1 isolates. Maximum likelihood 700 trees based on E gene (A) or complete genome (B) sequences. Sequences from GenBank 701 are in black font and sequences generated in this study are in color. Blue and red fonts 702 indicate isolates used in experiment 1 and 2, respectively. All horizontal branch lengths 703 are drawn to scale, with bootstrap support values shown next to relevant nodes. 704 705 Figure 3. Variation of artificial DENV-1 blood meal titers in vector competence 706 **assays.** Infectious titers of the artificial blood meals measured by plague assay are shown 707 for each isolate as a function of their date of isolation and phylogenetic group. Each 708 isolate was tested twice in two experimental blocks (for details see Materials and 709 Methods). Lines represents linear y=f(x) regressions to give a graphical indication of

710 temporal trends. Lower lines correspond to experimental block 1; upper lines correspond 711 to experimental block 2.

712

725



and new clades at 7 and 14 days post-blood meal. Error bars indicate the standard errors 726 of the means. Statistical significance of difference between clades is given by P-values

727 derived from a complete multifactorial analysis of variance accounting for the effects of

728 infectious dose (blood meal titer), experiment, block, clade and isolates; the analysis only

729 includes mosquitoes with a disseminated infection.







- Early clade block 1
- New clade block 1
- Early clade block 2
- New clade block 2



