



Evolutionary dynamics of dengue virus populations within the mosquito vector

Louis Lambrechts, Sebastian Lequime

► To cite this version:

Louis Lambrechts, Sebastian Lequime. Evolutionary dynamics of dengue virus populations within the mosquito vector. *Current Opinion in Virology*, 2016, 21, pp.47-53. <10.1016/j.coviro.2016.07.013>. <hal-01445721>

HAL Id: hal-01445721

<https://hal.science/hal-01445721v1>

Submitted on 14 Feb 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Evolutionary dynamics of dengue virus populations within the mosquito vector

Louis Lambrechts^{a,b,} and Sebastian Lequime^{a,b,c}*

^aInsect-Virus Interactions Group, Department of Genomes and Genetics, Institut Pasteur, Paris, France

^bCentre National de la Recherche Scientifique, Unité de Recherche Associée 3012, Paris, France

^cUniversité Pierre et Marie Curie, Cellule Pasteur UPMC, Paris, France

*Corresponding author. Address: Insect-Virus Interactions, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France. E-mail address: louis.lambrechts@pasteur.fr

Abstract

To date, dengue virus evolution has mainly been addressed by studies conducted at the between-host level. Like other pathogens with high mutation rate and rapid replication, dengue viruses also evolve during the course of an infection. Over the last few years, the advent of deep-sequencing technologies has facilitated studies of dengue virus populations at the within-host level. Here, we review recent advances on the evolutionary dynamics of dengue virus populations within their mosquito vector. We discuss how identifying the evolutionary forces acting on dengue virus populations within the mosquito can shed light on the processes underlying vector-virus interactions and the evolution of epidemiologically relevant traits.

Introduction

Dengue viruses (DENV) are mosquito-borne RNA viruses in the genus *Flavivirus* (family Flaviviridae) that cause more human disease than any other arthropod-borne (arbo) virus [1]. They consist of four serotypes (DENV-1 to -4) that are phylogenetically related [2] and loosely antigenically distinct [3]. Human DENV originate from sylvatic transmission cycles that occur between non-human primates and arboreal mosquitoes [4]. They emerged and became endemic in the human population in the last few hundred years [2]. Today, DENV are transmitted among human hosts by anthropophilic mosquito vectors, primarily *Aedes aegypti* [5].

Over the last three decades, phylogenetic and molecular evolution analyses based on viral genomic sequences have provided valuable insights into DENV evolutionary history and epidemiology [6]. It is noteworthy that this large body of work on various aspects of DENV biology such as the origin, spatio-temporal transmission dynamics, and pathogenesis has mainly been conducted at the between-host level. In other words, viral genomic sequences have generally been analyzed by considering a single genome per human or mosquito host sampled, which makes the implicit assumption that a single DENV genome adequately represents a given infection.

Due to their exceptionally high mutation rate – about a million times greater than that of vertebrates [7] – RNA viruses typically exist within their hosts as a heterogeneous population of related genetic variants, often referred to as a viral quasispecies [8]. The within-host genetic diversity of DENV was initially demonstrated by reverse transcription of viral RNA purified from individual human plasma samples, followed by cloning and sequencing of multiple PCR amplicons [9,10]. The majority of subsequent studies focused on the human host [11-14], although the within-host genetic diversity of DENV populations was also confirmed in mosquitoes [15,16]. Within-host DENV genetic diversity was initially reported to be lower in mosquitoes than in human patients [16], but this observation did not hold in later studies [15,17].

Early studies of within-host DENV genetic diversity based on clonal sequencing typically examined partial genome sequences, such as the viral envelope gene, or a limited number of complete genome sequences. With the advent of high-throughput sequencing technologies,

genome-wide surveys progressively became the standard approach [13,14,18,19], with some exceptions [20]. The primary aim of these studies was to estimate the extent of within-host DENV genetic diversity, and possibly correlate it with relevant clinical, epidemiological or phylogenetic features. For example, a comprehensive study of within-host DENV-2 genetic diversity in Nicaragua revealed non-uniform patterns across viral genes, variation across phylogenetic lineages, and some degree of relationship with between-host genetic diversity [13]. One unresolved question is whether dengue disease severity in humans is associated with the level of within-host DENV genetic diversity, as was reported in some instances [12] but not others [13,14].

Only during the last two years did studies begin to monitor the evolutionary dynamics of DENV populations at the within-host level [17,18,21-23]. The abundant genetic diversity of RNA virus populations, coupled with short generation times, fuels their rapid evolution over the course of a single infection. Combining deep sequencing of within-host viral populations (Figure 1) with computational and statistical methods allows empirical analyses of their evolutionary dynamics [24]. Viral evolutionary analyses rely on the observed patterns of genetic diversity to infer the underlying evolutionary processes [25]. Evolutionary forces acting on within-host viral populations are not fundamentally different from those acting on any population. They include genetic mechanisms (i.e., mutation and recombination), demographic processes (i.e., genetic drift) and natural selection.

Here, we review our current knowledge on the within-host evolutionary dynamics of DENV, focusing on the infection of mosquito vectors because they represent the bulk of published studies to date. We illustrate how the analysis of within-host evolutionary dynamics can provide important insights into DENV-vector interactions and DENV epidemiology.

Within-host evolution of DENV populations

To our knowledge, only five studies published to date monitored DENV genetic diversity at the within-host level based on sequential observations of the same viral population. Rodriguez-Roche et al. examined patterns of within-host genetic diversity in human serum samples collected during a DENV-3 epidemic in Cuba [18]. Although this observation was based on a single dengue patient, they detected significant changes in the genetic composition

of the within-host viral population sampled two and four days after fever onset. Plummer et al. monitored the evolution of DENV-2 genetic diversity during several passages in a mouse model to evaluate the effect of an antiviral molecule [22]. The antiviral treatment resulted in a higher number of viral genetic variants and signals of positive selection. In addition, viral populations passaged in both the antiviral-treated mice and the control mice differed from the input virus, indicating that evolution also took place independently of the antiviral treatment.

Sessions et al. examined DENV-1 genetic diversity in twelve paired human sera collected early and late (i.e., four days apart) during the course of the infection, and in both *Ae. aegypti* and *Aedes albopictus* mosquitoes ten days after intrathoracic inoculation with the same human serum samples [23]. They detected mutation hotspots and coldspots in the viral genome, some of which were independent of the host type. In addition, there was evidence for frequent reversion of low-frequency variants during human-to-mosquito transfer, and some degree of mosquito species-specific evolution. Sim et al. also tracked changes in within-host DENV-1 genetic diversity during human-to-mosquito transmission, but using a more natural setting [17]. Twelve viremic dengue patients from Vietnam were experimentally exposed to naïve *Ae. aegypti* mosquitoes, and three blood-fed mosquitoes per patient were analyzed after two weeks of extrinsic incubation. Comparison of within-host DENV genetic diversity in matched human and mosquito samples revealed substantial changes in the variant repertoire between venous blood and the organs of mosquitoes that became infected by feeding on the corresponding person. The majority of genetic variants were lost upon transition from venous blood to mosquito abdomen, as well as from mosquito abdomen to salivary glands. Nevertheless, several measures of genetic diversity per sample were similar between human plasma, mosquito abdomens and mosquito salivary glands, indicating that the overall level of within-host genetic diversity was maintained.

A study that our research group recently conducted investigated the evolutionary forces underlying within-host DENV evolution in different genotypes of their mosquito vector *Ae. aegypti* [21]. We used deep sequencing to monitor the genetic diversity of full-length viral genomes during mosquito infection by a wild-type DENV-1 isolate (Figure 2). Based on neutral or quasi-neutral genetic markers to rule out a confounding effect of natural selection, we detected a stochastic reduction in population size at initial midgut infection. We estimated that infection was randomly initiated by tens of viral genomes, two orders of magnitude less than the infectious dose of virus ingested by an individual mosquito in our experiment. The

observed level of genetic diversity increased substantially during population expansion in the midgut without evidence for positive selection. Importantly, the observed amount of viral genetic diversity differed among mosquito genetic backgrounds, despite a similar census population size. Although additional investigations are necessary to determine their exact nature, this study thus pointed to a role for host genetic factors in the genetic breadth of within-host DENV genetic diversity. This result extends to the host-genotype level the recent finding that within-host West Nile virus genetic diversity is host-species dependent [26].

Inferring DENV-vector interactions from within-host viral evolutionary dynamics

The above studies illustrate how the evolutionary dynamics of within-host viral populations can provide insights into DENV-vector interactions. Fluctuations in viral population size caused by anatomical barriers, for instance, can be inferred by comparing the genetic variance before and after traversal of the barrier [27,28]. Such methods use neutral or quasi-neutral genetic variants to estimate the effective population size (N_e), which is the size of an idealized population that would drift at the same rate as the observed population. The relatively tight population bottleneck observed at initial infection of the mosquito midgut [21] indicates that DENV likely establish infection in a small number of cells, as was observed for other arboviruses [29]. One caveat of methods based on N_e estimation is that the neutrality assumption, even for synonymous substitutions, is difficult to verify for RNA viruses [30]. Nevertheless, these methods allow demographic analyses of wild-type viral populations that would otherwise require artificial variants mixtures [31], marked viral clones [32], or viral replicons expressing a reporter protein [33].

Measures of natural selection can also shed light on the processes at play during DENV-vector interactions. The strength and direction of natural selection are commonly assessed by computing the ratio of non-synonymous to synonymous changes (d_N/d_S). A d_N/d_S ratio of one means that non-synonymous substitutions occur at the same rate as synonymous substitutions and is interpreted as evidence for neutral evolution. A d_N/d_S ratio greater than one implies positive selection (i.e., driving change), whereas a d_N/d_S ratio smaller than one indicates purifying selection (i.e., opposing change). Analysis of the d_N/d_S ratio can only be applied to coding regions, it cannot detect certain forms of natural selection [34] and has several limitations at the within-population level [35]. However, calculating the d_N/d_S ratio can be

useful to compare the selection regime in different host compartments or different host types. The within-host environment is typically heterogeneous (i.e., different cell types) and spatially structured (i.e., organs), which can lead to the emergence of fitness trade-offs [36]. Such within-host trade-offs remain to be explored in the case of DENV.

Host immunity is expected to represent a major selective pressure on viral populations, and mosquito hosts are no exception. Antiviral immunity in insects consists of several arms that include RNA interference (RNAi), apoptosis, the ubiquitin proteasome pathway, autophagy and the heat-shock response [37]. RNAi is often described as the major antiviral immune response of mosquitoes against arboviral infections [38]. Because RNAi results in viral RNA degradation in a sequence-specific manner, there is potential for within-host co-evolutionary dynamics between viral populations and the RNAi response. Under this hypothetical scenario, rare viral variants are selectively advantaged if their sequence is sufficiently divergent to escape the RNAi response targeted at the dominant viral sequence. Consistent with this hypothesis, a study of West Nile virus infection in *Culex* mosquitoes showed that intense RNAi targeting of the viral genome was associated with positive selection [39]. As the escape variants rise in frequency, they are increasingly targeted by the RNAi response, allowing other rare variants to be selected. This mechanism of negative frequency-dependent selection, whereby allele fitness decreases as a function of the relative allele frequency in the population, is an example of balancing selection (that cannot be detected with the d_N/d_S ratio).

The shape of the viral phylogeny at the within-host level may also provide indications on within-host processes. It has been suggested that different within-host processes may result in a different phylogeny shape (i.e., different tree topology and distribution of branch lengths), so that these mechanisms could be inferred from time series of within-host viral genetic diversity [36]. For example, resource-mediated and immune-mediated competition are expected to result in different trajectories of within-host evolution [36]. Thus, analysis of the shape of viral phylogenies at the within-host level could help to elucidate the mechanisms of DENV-vector interactions. One technical difficulty to overcome is haplotype phasing (i.e., resolving the combination of nucleotide variants carried by one viral genome) using short-read sequence data, for which existing programs perform poorly when sequence divergence is low [40].

Connecting within-host and between-host DENV evolutionary dynamics

Dengue epidemiology (i.e., at the between-host level) can also benefit from a better understanding of within-host evolutionary dynamics because within-host and between-host levels are connected. Perhaps the most eloquent example of this connection is the long-term transmission of defective DENV genomes in nature. A DENV-1 lineage that acquired a stop-codon mutation in the envelope gene was detected in both humans and mosquitoes over a period of 18 months in Myanmar [15]. Such defective genomes, in which large sections have been deleted, have a replication advantage over the full-length parental genomes but they are only replicated by complementation in the presence of co-infecting functional viruses [41]. Defective interfering particles compete with functional viruses [42] but may also contribute to infection persistence [43]. Maintenance of a defective DENV lineage over multiple transmission cycles between humans and mosquitoes implies that population bottlenecks are not severe enough to prevent complementation by a functional DENV lineage, consistent with the estimated bottleneck size of tens of DENV genomes at initial mosquito infection [21]. A modeling study accounting for both within-host and between-host dynamics showed that co-transmission of defective and functional DENV can potentiate epidemic spread [44]. Thus, within-host interactions between viral variants (i.e., complementation) may provide a pathway to viral emergence.

On a longer time scale, accounting for within-host evolutionary dynamics may help to predict the between-host viral evolutionary rate [45,46]. In the case of arboviruses, it was observed that their evolutionary rate is on average slower than that of directly-transmitted RNA viruses [47]. The fitness trade-off hypothesis postulates that arbovirus alternation between disparate host types (i.e., vertebrate and invertebrate) constraints adaptation and slows down evolution, but this hypothesis has only received partial support from empirical studies [48]. Another important feature of the arbovirus life cycle are the recurrent population bottlenecks that occur during transmission [49]. These bottlenecks may act to slow down arbovirus evolution independently of host alternation. Presumably, rare adaptive mutations are lost by genetic drift, whereas more frequent deleterious mutations are efficiently removed by purifying selection during phases of within-host population expansion. The combined action of recurrent population bottlenecks and within-host purifying selection may thus promote evolutionary conservation of arboviral genomes.

230

231 **Conclusions**

232

233 Only a handful of studies investigated the dynamics of within-host DENV genetic diversity to
234 date. Most of these studies used a descriptive approach to correlate the extent of within-host
235 viral genetic diversity with epidemiologically relevant features. This is a time of great
236 opportunities to carry out longitudinal studies of within-host DENV evolutionary dynamics
237 that will go beyond the mere description of genetic diversity. By combining high-throughput
238 sequencing data with computational and statistical methods, within-host evolutionary analysis
239 of DENV has the potential to illuminate within-host processes and provide important insights
240 into epidemiological dynamics. Another critical aspect for future research will be to move
241 from the analysis of patterns of DENV genetic diversity to the analysis of phenotypic traits
242 underlying DENV fitness (e.g., drug resistance, replication rate, extrinsic incubation period).
243 This will require the alliance of robust within-host viral evolutionary analysis and carefully
244 designed experiments of viral reverse genetics.

Acknowledgements

The authors thank Claudine Leroy for providing the mosquito sketch included in Figure 2, and Samuel Alizon for insightful discussions. The authors were supported by the French Government's Investissement d'Avenir program Laboratoire d'Excellence Integrative Biology of Emerging Infectious Diseases (grant ANR-10-LABX- 62-IBEID), and the City of Paris Emergence(s) program in Biomedical Research. The funding sources had no role in study design or decision to submit the article for publication.

References and recommended reading

Papers of particular interest, published within the period of the review, have been highlighted as:

* of special interest

** of outstanding interest

1. Guzman MG, Harris E: **Dengue**. *Lancet* 2015, **385**:453-465.
2. Holmes EC, Twiddy SS: **The origin, emergence and evolutionary genetics of dengue virus**. *Infect Genet Evol* 2003, **3**:19-28.
3. Katzelnick LC, Fonville JM, Gromowski GD, Bustos Arriaga J, Green A, James SL, Lau L, Montoya M, Wang C, VanBlargan LA, et al.: **Dengue viruses cluster antigenically but not as discrete serotypes**. *Science* 2015, **349**:1338-1343.
4. Vasilakis N, Weaver SC: **The history and evolution of human dengue emergence**. *Adv Virus Res* 2008, **72**:1-76.
5. Lambrechts L, Scott TW, Gubler DJ: **Consequences of the expanding global distribution of *Aedes albopictus* for dengue virus transmission**. *PLoS Negl Trop Dis* 2010, **4**:e646.
6. Weaver SC, Vasilakis N: **Molecular evolution of dengue viruses: contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease**. *Infect Genet Evol* 2009, **9**:523-540.
7. Duffy S, Shackelton LA, Holmes EC: **Rates of evolutionary change in viruses: patterns and determinants**. *Nat Rev Genet* 2008, **9**:267-276.
8. Llaure AS, Andino R: **Quasispecies theory and the behavior of RNA viruses**. *PLoS Pathog* 2010, **6**:e1001005.
9. Wang WK, Lin SR, Lee CM, King CC, Chang SC: **Dengue type 3 virus in plasma is a population of closely related genomes: quasispecies**. *J Virol* 2002, **76**:4662-4665.
10. Wang WK, Sung TL, Lee CN, Lin TY, King CC: **Sequence diversity of the capsid gene and the nonstructural gene NS2B of dengue-3 virus in vivo**. *Virology* 2002, **303**:181-191.
11. Chao DY, King CC, Wang WK, Chen WJ, Wu HL, Chang GJ: **Strategically examining the full-genome of dengue virus type 3 in clinical isolates reveals its mutation spectra**. *Virol J* 2005, **2**:72.

12. Descloux E, Cao-Lormeau VM, Roche C, De Lamballerie X: **Dengue 1 diversity and microevolution, French Polynesia 2001-2006: connection with epidemiology and clinics.** *PLoS Negl Trop Dis* 2009, **3**:e493.
13. Parameswaran P, Charlebois P, Tellez Y, Nunez A, Ryan EM, Malboeuf CM, Levin JZ, Lennon NJ, Balmaseda A, Harris E, et al.: **Genome-wide patterns of intrahuman dengue virus diversity reveal associations with viral phylogenetic clade and interhost diversity.** *J Virol* 2012, **86**:8546-8558.
- * This comprehensive analysis of within-host dengue virus genetic diversity in human serum samples pointed out correlations with clinical, epidemiological and virological features.
14. Thai KT, Henn MR, Zody MC, Tricou V, Nguyet NM, Charlebois P, Lennon NJ, Green L, de Vries PJ, Hien TT, et al.: **High-resolution analysis of intrahost genetic diversity in dengue virus serotype 1 infection identifies mixed infections.** *J Virol* 2012, **86**:835-843.
15. Aaskov J, Buzacott K, Thu HM, Lowry K, Holmes EC: **Long-term transmission of defective RNA viruses in humans and Aedes mosquitoes.** *Science* 2006, **311**:236-238.
- **This landmark study combined assessment of within-host dengue virus genetic diversity in humans and mosquitoes and phylogenetic analyses to demonstrate sustained transmission of a viral lineage associated with a truncated genome.
16. Lin SR, Hsieh SC, Yueh YY, Lin TH, Chao DY, Chen WJ, King CC, Wang WK: **Study of sequence variation of dengue type 3 virus in naturally infected mosquitoes and human hosts: implications for transmission and evolution.** *J Virol* 2004, **78**:12717-12721.
17. Sim S, Aw PP, Wilm A, Teoh G, Hue KD, Nguyen NM, Nagarajan N, Simmons CP, Hibberd ML: **Tracking Dengue Virus Intra-host Genetic Diversity during Human-to-Mosquito Transmission.** *PLoS Negl Trop Dis* 2015, **9**:e0004052.
- * This analysis of within-host dengue virus genetic diversity during human-to-mosquito transmission revealed a profound reshuffling of the viral variant repertoire.
18. Rodriguez-Roche R, Blanc H, Borderia AV, Diaz G, Henningsson R, Gonzalez D, Santana E, Alvarez M, Castro O, Fontes M, et al.: **Increasing Clinical Severity**

- 320 **during a Dengue Virus Type 3 Cuban Epidemic: Deep Sequencing of Evolving**
321 **Viral Populations.** *J Virol* 2016, **90**:4320-4333.
- 322 19. Romano CM, Lauck M, Salvador FS, Lima CR, Villas-Boas LS, Araujo ES, Levi JE,
323 Pannuti CS, O'Connor D, Kallas EG: **Inter- and intra-host viral diversity in a large**
324 **seasonal DENV2 outbreak.** *PLoS ONE* 2013, **8**:e70318.
- 325 20. Choudhury MA, Lott WB, Banu S, Cheng AY, Teo YY, Ong RT, Aaskov J: **Nature and**
326 **Extent of Genetic Diversity of Dengue Viruses Determined by**
327 **Pyrosequencing.** *PLoS ONE* 2015, **10**:e0142473.
- 328 21. Lequime S, Fontaine A, Ar Gouilh M, Moltini-Conclois I, Lambrechts L: **Genetic Drift,**
329 **Purifying Selection and Vector Genotype Shape Dengue Virus Intra-host Genetic**
330 **Diversity in Mosquitoes.** *PLoS Genet* 2016, **12**:e1006111.
- 331 **This study identified the main evolutionary forces at play during mosquito infection by
332 dengue viruses, and uncovered the influence of host genotype on the amount of
333 within-host viral genetic diversity.
- 334
- 335 22. Plummer E, Buck MD, Sanchez M, Greenbaum JA, Turner J, Grewal R, Klose B,
336 Sampath A, Warfield KL, Peters B, et al.: **Dengue Virus Evolution under a Host-**
337 **Targeted Antiviral.** *J Virol* 2015, **89**:5592-5601.
- 338 23. Sessions OM, Wilm A, Kamaraj US, Choy MM, Chow A, Chong Y, Ong XM, Nagarajan
339 N, Cook AR, Ooi EE: **Analysis of Dengue Virus Genetic Diversity during Human**
340 **and Mosquito Infection Reveals Genetic Constraints.** *PLoS Negl Trop Dis* 2015,
341 **9**:e0004044.
- 342 24. Nelson CW, Hughes AL: **Within-host nucleotide diversity of virus populations:**
343 **insights from next-generation sequencing.** *Infect Genet Evol* 2015, **30**:1-7.
- 344 25. Pybus OG, Rambaut A: **Evolutionary analysis of the dynamics of viral infectious**
345 **disease.** *Nat Rev Genet* 2009, **10**:540-550.
- 346 26. Grubaugh ND, Weger-Lucarelli J, Murrieta RA, Fauver JR, Garcia-Luna SM, Prasad AN,
347 Black WC, Ebel GD: **Genetic Drift during Systemic Arbovirus Infection of**
348 **Mosquito Vectors Leads to Decreased Relative Fitness during Host Switching.**
349 *Cell Host Microbe* 2016, **19**:481-492.
- 350 27. Thebaud G, Michalakakis Y: **Comment on "Large Bottleneck Size in Cauliflower Mosaic**
351 **Virus Populations during Host Plant Colonization" by Monsion et al. (2008).**
352 *PLoS Pathog* 2016, **12**:e1005512.

28. Monsion B, Froissart R, Michalakakis Y, Blanc S: **Large bottleneck size in Cauliflower Mosaic Virus populations during host plant colonization.** *PLoS Pathog* 2008, **4**:e1000174.
29. Smith DR, Adams AP, Kenney JL, Wang E, Weaver SC: **Venezuelan equine encephalitis virus in the mosquito vector *Aedes taeniorhynchus*: infection initiated by a small number of susceptible epithelial cells and a population bottleneck.** *Virology* 2008, **372**:176-186.
30. Cuevas JM, Domingo-Calap P, Sanjuan R: **The fitness effects of synonymous mutations in DNA and RNA viruses.** *Mol Biol Evol* 2012, **29**:17-20.
31. Ciota AT, Ehrbar DJ, Van Slyke GA, Payne AF, Willsey GG, Viscio RE, Kramer LD: **Quantification of intrahost bottlenecks of West Nile virus in *Culex pipiens* mosquitoes using an artificial mutant swarm.** *Infect Genet Evol* 2012, **12**:557-564.
32. Forrester NL, Guerbois M, Seymour RL, Spratt H, Weaver SC: **Vector-borne transmission imposes a severe bottleneck on an RNA virus population.** *PLoS Pathog* 2012, **8**:e1002897.
33. Gutierrez S, Thebaud G, Smith DR, Kenney JL, Weaver SC: **Demographics of natural oral infection of mosquitos by Venezuelan equine encephalitis virus.** *J Virol* 2015, **89**:4020-4022.
34. Yang Z, Bielawski JP: **Statistical methods for detecting molecular adaptation.** *Trends Ecol Evol* 2000, **15**:496-503.
35. Kryazhimskiy S, Plotkin JB: **The population genetics of dN/dS.** *PLoS Genet* 2008, **4**:e1000304.
36. Alizon S, Luciani F, Regoes RR: **Epidemiological and clinical consequences of within-host evolution.** *Trends Microbiol* 2011, **19**:24-32.
37. Marques JT, Imler JL: **The diversity of insect antiviral immunity: insights from viruses.** *Curr Opin Microbiol* 2016, **32**:71-76.
38. Blair CD, Olson KE: **Mosquito immune responses to arbovirus infections.** *Curr Opin Insect Sci* 2014, **3**:22-29.
39. Brackney DE, Beane JE, Ebel GD: **RNAi targeting of West Nile virus in mosquito midguts promotes virus diversification.** *PLoS Pathog* 2009, **5**:e1000502.
40. Schirmer M, Sloan WT, Quince C: **Benchmarking of viral haplotype reconstruction programmes: an overview of the capacities and limitations of currently available programmes.** *Brief Bioinform* 2014, **15**:431-442.

41. Li D, Lott WB, Lowry K, Jones A, Thu HM, Aaskov J: **Defective interfering viral particles in acute dengue infections.** *PLoS ONE* 2011, **6**:e19447.
42. Huang AS, Baltimore D: **Defective viral particles and viral disease processes.** *Nature* 1970, **226**:325-327.
43. Holland JJ, Villarreal LP: **Persistent noncytotoxic vesicular stomatitis virus infections mediated by defective T particles that suppress virion transcriptase.** *Proc Natl Acad Sci U S A* 1974, **71**:2956-2960.
44. Ke R, Aaskov J, Holmes EC, Lloyd-Smith JO: **Phylogenetic analysis of the emergence and epidemiological impact of transmissible defective dengue viruses.** *PLoS Pathog* 2013, **9**:e1003193.
- * This study combined dynamical modeling and phylogenetic analyses to highlight the link between within-host dynamics and epidemiological patterns in the case of defective dengue viruses.
45. Scholle SO, Ypma RJ, Lloyd AL, Koelle K: **Viral substitution rate variation can arise from the interplay between within-host and epidemiological dynamics.** *Am Nat* 2013, **182**:494-513.
46. Peck KM, Chan CHS, Tanaka M: **Connecting within-host dynamics to the rate of viral molecular evolution.** *Virus Evol* 2015, **1**:vev013.
47. Jenkins GM, Rambaut A, Pybus OG, Holmes EC: **Rates of molecular evolution in RNA viruses: a quantitative phylogenetic analysis.** *J Mol Evol* 2002, **54**:156-165.
48. Coffey LL, Forrester N, Tsetsarkin K, Vasilakis N, Weaver SC: **Factors shaping the adaptive landscape for arboviruses: implications for the emergence of disease.** *Future Microbiol* 2013, **8**:155-176.
49. Forrester NL, Coffey LL, Weaver SC: **Arboviral bottlenecks and challenges to maintaining diversity and fitness during mosquito transmission.** *Viruses* 2014, **6**:3991-4004.
50. Ellefson JW, Gollihar J, Shroff R, Shivram H, Iyer VR, Ellington AD: **Synthetic evolutionary origin of a proofreading reverse transcriptase.** *Science* 2016, **352**:1590-1593.
51. Cruz CD, Torre A, Troncos G, Lambrechts L, Leguia M: **Targeted full-genome amplification and sequencing of dengue virus types 1-4 from South America.** *J Virol Methods* 2016, **235**:158-167.

52. Isakov O, Borderia AV, Golan D, Hamenahem A, Celniker G, Yoffe L, Blanc H, Vignuzzi M, Shomron N: **Deep sequencing analysis of viral infection and evolution allows rapid and detailed characterization of viral mutant spectrum.** *Bioinformatics* 2015, **31**:2141-2150.

Figure legends

Figure 1: Deep-sequencing analysis of within-host viral populations. (1) RNA is extracted from the sample, purified and reverse transcribed into complementary DNA (cDNA) using random hexamers or a specific reverse primer located at 3' end of the viral genome. Reverse transcription is the most error-prone step of the library preparation because the reverse transcriptase (RT) lacks proofreading activity; however, recent advances in understanding RT activity may lead to the development of proofreading RT and increased fidelity of this step [50]. Double-stranded DNA (dsDNA) is obtained by synthesis of the second strand or by high-fidelity PCR amplification. Samples with low template concentration usually require PCR amplification using a variable number of overlapping amplicons to cover the viral genome [51]. dsDNA is prepared for sequencing through indexing (for multiplexing purposes) and ligation of sequencing adapters. This step usually includes or is followed by another round of PCR amplification. Minimizing the number of PCR cycles during library preparation is critical for data quality because each PCR cycle can introduce errors that are difficult to discriminate from true low-frequency variants, even when a high-fidelity DNA polymerase is used. (2) The dsDNA library is deeply sequenced (i.e., a given nucleotide position is sequenced hundreds to thousands of times per sample) on a high-throughput sequencing platform. Illumina has been the most popular sequencing technology in recent years [17,21,23]. (3) Sequencing reads are checked for quality (i.e., low-quality nucleotides are trimmed) and PCR duplicates may be removed. (4) Reads are aligned to the reference viral genome, which can be predetermined or assembled *de novo* using the newly generated sequence data. (5) Based on the alignment of sequencing reads and their quality score, variant-calling algorithms detect nucleotide variants for each position and estimate their relative frequency [52]. The variant detection threshold primarily depends on the sequencing depth, with deeper sequencing resulting in higher sensitivity. Finally, nucleotide variants and their frequencies are used for analyses of viral population genetics.

Figure 2: Dynamics of DENV genetic diversity within the mosquito *Aedes aegypti*. The mosquito digestive tract (in red) is infected following a DENV infectious blood meal. A sharp population drop occurs at initial midgut infection [17,21] followed by intense replication resulting in a large population size and increased level of genetic diversity compared to the input virus. DENV release into the hemocoel (i.e., the body cavity containing the

hemolymph) leads to systemic dissemination and infection of secondary organs (in gray), including salivary glands (in blue). DENV genetic diversity in the hemocoel is yet to be documented, and it is unclear to what extent viral populations undergo repeated population bottlenecks during systemic dissemination as was recently shown for West Nile virus [26]. Salivary gland infection is necessary for virus transmission to a new host. DENV populations in salivary glands are genetically diverse and may contain genetic variants that are different from the parental consensus sequence at the individual mosquito level. The minute amounts of DENV RNA that can be recovered from saliva samples are a hurdle to assess the potentially transmitted viral genetic diversity, which is currently unknown. Viral loads expressed in RNA copies and within-host genetic diversity levels shown in the figure are based on data from *Ae. aegypti* isofemale line CD in Lequime et al. [21].

Figure 1
Click here to download Figure: Figure1.eps

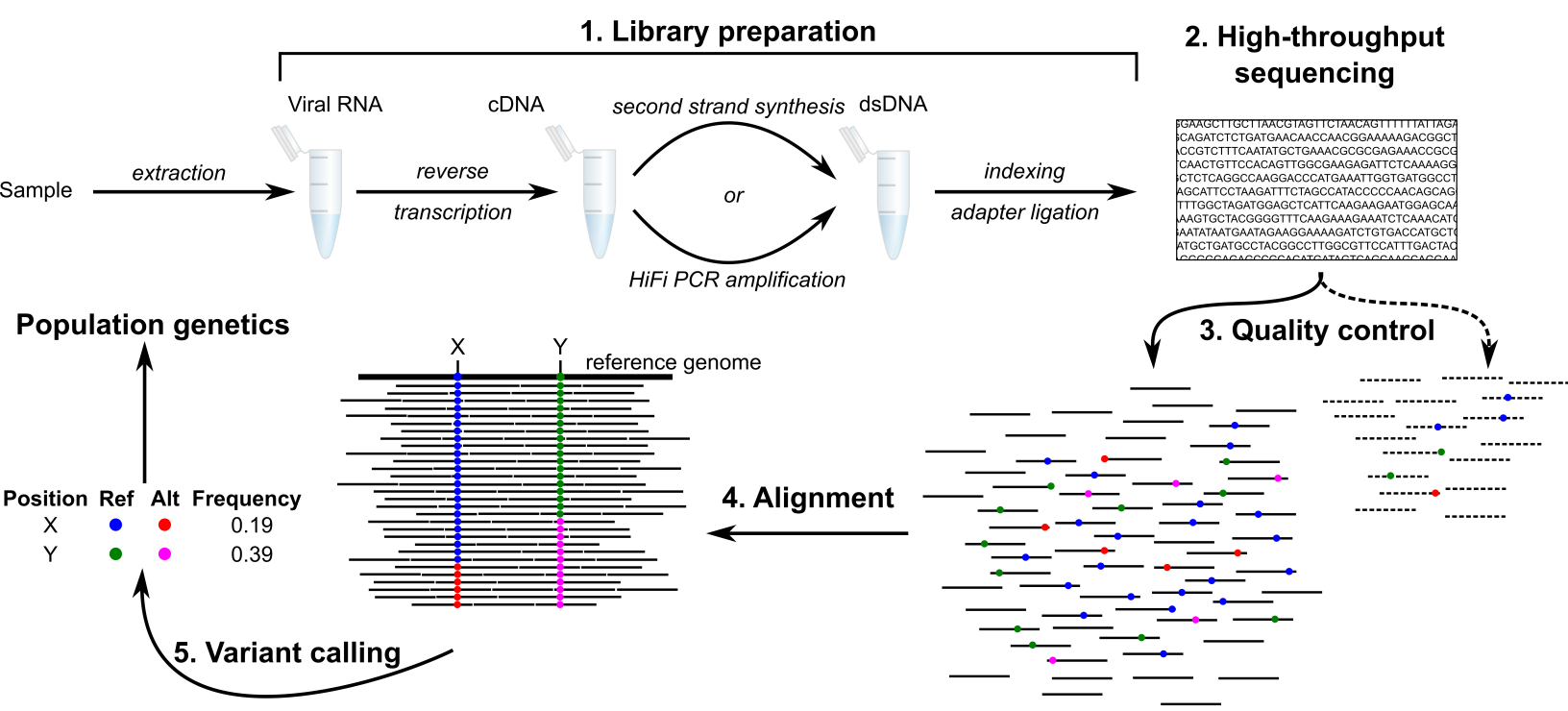


Figure 2
[Click here to download Figure: Figure2.eps](#)

