Substantial Deceleration of Adaptation of HIV-1 Within 1,500 Generations in an Experimental Evolution: A Genomic Perspective

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Abstract

Numerous experimental evolution studies have suggested that adaptation rate of microbial populations evolving in stable environments decline over time. Despite the generality of this phenomenon across different domains of life, the timing and magnitude of decline in adaptation can vary greatly based on the idiosyncrasies of the biological system. To investigate the characteristics of adaptation deceleration in a fast-evolving virus, we propagated HIV-1 in two human T-cell lines (MT-2 and MT-4) for approximately 4.8 years and tracked its genome evolution through next-generation sequencing. The curated sequencing data covering the whole-genome can be accessed and explored via LTEEviz, an interactive web application. Time-resolved sequencing data uncovered that despite constant fixation rate of 0.085 (MT-2) and 0.042 (MT-4) mutations per generation, the fixation kinetics of adaptive mutations changed considerably over time. The rate of fixation of adaptive parallel mutations decreased by 44% per 300 generations, while their conferred fitness gain decreased by 27% (MT-2) and 18% (MT-4) per every added adaptive mutation in their genetic background. The early and substantial deceleration of adaptation in our HIV-1 populations can, at least in part, be explained by diminishing gains of adaptive mutations. Furthermore, we identified genomic patterns consistent with a hard selective sweep that occurred in one population later in the experiment. Together, our results confirm that HIV-1 genomic evolution is characterized by a swift and substantial deceleration of adaptation, while also revealing that episodes of positive selection can occur beyond the initial adaptive phase.

Key words: adaptive evolution, deceleration of adaptation, parallel evolution, HIV-1, long-term evolution experiment (LTEE)

Introduction

Decades of evolutionary research, spanning from theoretical models to empirical studies, have indicated that the fitness of evolving microbial populations in a stable environment effectively reaches a plateau[1–3]. Despite the predictable decline in the rate of adaptation in constant environments, the trajectories and timescales by which populations approach the fitness optimum can vary greatly[3–6]. Stochasticity in evolutionary processes permits populations to take unique paths through genotype space[7], while the rate of adaptation is influenced by several factors such as availability of genetic variation[8], the demographic properties of populations[9], and the topology of the fitness landscape[10]. Thus, a full understanding of the mode and tempo of adaptation and its determinants requires examination of adaptive dynamics in a wide range of organisms.

One approach to studying adaptive dynamics of evolving populations is to probe patterns of polymorphism and divergence at the nucleotide level[11]. These patterns are shaped by the interplay of multiple evolutionary forces, including selection as the propellant of adaptation[12]. When populations approach an adaptive peak, the influence of positive selection on genome evolution wanes[13], while other background evolutionary forces continue to operate unabated[14,15]. Hence, identifying and gauging footprints of positive selection in the genome, or lack thereof, can be informative about the underlying adaptive dynamics[16].

To elucidate the adaptive dynamics of evolving genomes, it is imperative to determine the genetic composition of evolving populations with high resolution and accuracy[17,18]. Efforts to generate such data from natural populations are often hindered by the inherent challenges of data acquisition and interpretation[19–21]. Long-term evolution experiments (LTEEs) offer a powerful alternative[22]. LTEEs enable us to replicate the evolution of biological (micro-)organisms in simplified, controlled environments[23]. Combined with advancing next-generation sequencing technologies, this framework promises to yield time-resolved genomic data, necessary to study the genetic basis of adaptation at finer resolution[24–26]. Furthermore, by employing different microorganisms, LTEEs allow us to systematically investigate potentially universal links between sequence evolution and adaptation.

Here, we describe in detail the dynamics of the genome evolution of human immunodeficiency virus type-1 (HIV-1) during the first five years of an LTEE. HIV-1 is a retrovirus renowned for rapid adaptation to diverse selective pressures, including host immune responses and antiviral drugs[27,28]. Its

efficient adaptability is mainly attributed to its exceptionally high mutation rate (10^{-4}-10^{-5} mutations per nucleotide per replication[29]), large population sizes[30], and relatively short generation time (1.2 days[31]). These features of the virus are also expected to facilitate rapid evolution under experimental settings, such as those in our cell cultures[32]. In addition to high adaptability, HIV-1's compact ~9.2 kb genome means that the virus can explore a sizable fraction of its fitness landscape within practical experimental time frames. In conjunction with a well-annotated genome and rich evolutionary literature, HIV-1 is therefore an exceptional model organism for studying the changing dynamics of adaptation based on genome evolution in a stable environment.

This work intends to build upon Bertels *et al.* (2019)[32], which reported striking levels of parallel evolution in four independently evolving HIV-1 populations using samples from the first 90 transfers of the same experiment. Here, we include additional sequencing and experimental data up to transfer 500, equivalent to ~1,500 generations and ~4.8 years of viral passaging, representing the longest-running evolution experiment of HIV-1. The analysis of the extended dataset uncovers a shift from increasing to decreasing parallelism across all populations. As parallel mutations that reach fixation are very likely adaptive[26,32–35]—a premise we support with multiple lines of evidence in our data—this transition marks a swift and substantial deceleration in adaptation. We ascribe this deceleration, at least partly, to the diminishing effects of adaptive mutations. Notably, we also describe genomic patterns consistent with a hard selective sweep in one population at later stages of the experiment, underscoring that episodes of adaptation may occur even after its deceleration. To promote further research, the curated genomic data can be accessed and explored via LTEEviz, an interactive web-based application.

Results

We passaged the ancestral HIV-1_{NL4-3} strain in the absence of immune responses or antiviral drugs in two different human T-cell lines (MT-2 and MT-4), with two replicates each (Fig. 1). These two cell lines are both HTLV-1-transformed, human leukemic T-cells, but are derived from two different donors and are associated with slightly different characteristics[36]. In each evolution line, 2 µl of the previous infected cell suspension was transferred into a new flask with fresh cell culture twice per week (500 times in total). Every 10th transfer, the genomic composition of populations was determined by next-generation

105 sequencing for each of the four evolutionary lines. Raw sequencing reads were processed via a data
106 pipeline (supplementary fig. S1).

107 We performed titration assays and qPCR to measure the key biological properties of the
108 evolving HIV-1 populations. The multiplicity of infection (MOI) of each transfer for MT-2 lines ranged
109 from 0.00018 to 0.0072 (median of 0.0016) and for MT-4 lines from 0.00039 to 0.027 (median of 0.0021)
110 (supplementary fig. S2). Since each fresh cell culture contained ~400,000 uninfected T-cells, that
111 roughly translates to a bottleneck size of 639 [IQR: 369,1109] and 842 [IQR: 369,2533] infectious units
112 in 2 µl of inoculum transferred in MT-2 and MT-4 lines, respectively. In concordance with lower overall
113 MOI in MT-2, effective population size estimates (N_e) were significantly lower for MT-2 compared to MT-
114 4 lines throughout the experiment (supplementary fig. S3, mean of 2.8 vs. 3.6 on log10 base).

115 Additionally, qPCR results show no considerable HIV-1 population growth or death throughout
116 the experiment (supplementary fig. S4, except for MT-2_1). Given a bottleneck fraction of 0.05%, we
117 therefore assumed that HIV-1 populations grew 2,000-fold between each subsequent transfer. As
118 previously justified in Bertels *et al.* (2019), we expect the infective viral offspring number (*R0*) to fall
119 between 12 and 44. Since the measurements of *R0* for HIV-1 both in vivo[37] and in cell culture[38] are
120 below the lower bound of this estimated range, we consider an *R0 of 12* for our HIV-1 populations. An
121 *R0* of 12 in turn mandates the completion of three generations during each incubation period of ~3.5
122 days, resulting in a generation time of ~1.2 days, which is within the range reported for HIV-1[39].
123 Furthermore, the harmonic mean of census population sizes for MT-2 and MT-4 throughout each
124 incubation period would be 4.37 and 5.06 on log10 scale (ratio of 4.8). Therefore, based on these
125 calculations and considering a half-life of 1.6 days for infected cells[40], the MOI is expected to approach
126 1 towards the end of the incubation period, thereby increasing the chance of coinfection and hence
127 recombination.

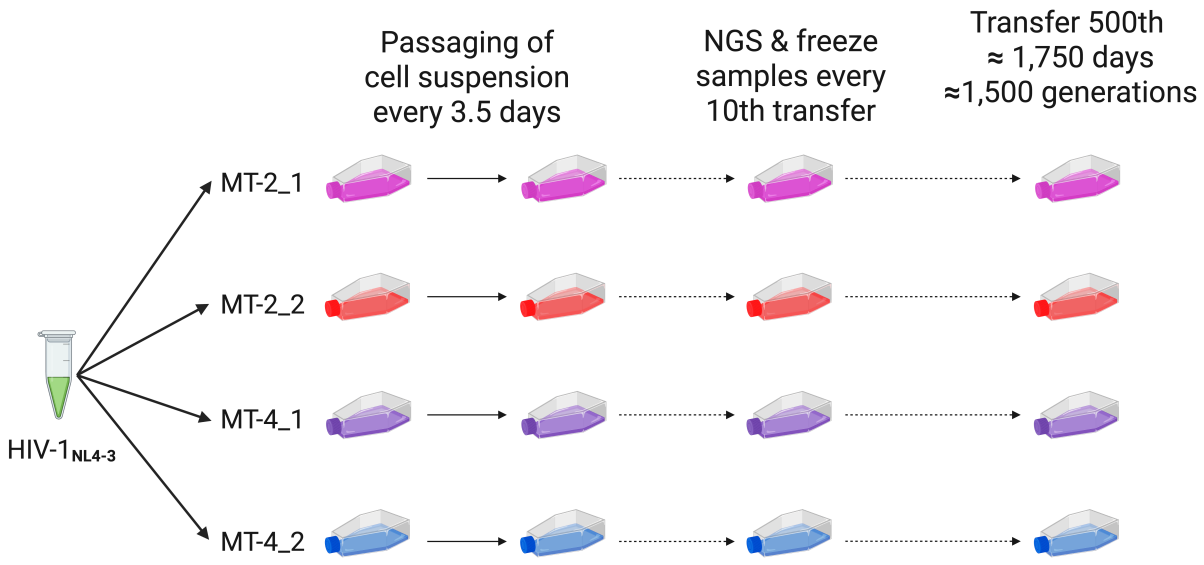


Fig. 1. Overview of long-term evolution experiment. The ancestral virus HIV-1$_{NL4-3}$ strain was passaged in four independent lines, with two biological replicates in each of two distinct human leukemia T-cell lines, MT-2 and MT-4. Every 3 or 4 days, 2 µl of infected cell suspension were transferred to a new flask containing uninfected cell culture. At every 10th transfer, cell-free supernatant of infected cell cultures were collected and used for full-length genome sequencing of evolving HIV-1 populations.

Increase in Genetic Diversity Is Slowing, Reaching Equilibrium in MT-2 Populations

To investigate the genome evolution of the four HIV-1 populations, we first quantified the genetic diversity using Shannon's index. The rate of increase in genetic diversity was initially comparable across hosts during the first 60 transfers of the experiment (Fig. 2A). However, the rate began to decline earlier in MT-2 than MT-4 lines. MT-4 populations continued to accumulate genetic diversity even towards the end of experiment, albeit at a reduced rate after transfer 120. In contrast, diversity levels in MT-2 lines nearly plateaued at around H = 0.022 bits by transfer 400.

Genetic diversity reaches an equilibrium when mutation and drift are in a balance[41]. In agreement with smaller effective population size estimates for MT-2 lines, we expect that stronger drift to be responsible for lower standing genetic variation observed in MT-2. First, similar rates of diversity increase during the first 60 transfers suggest that the mutation rates in HIV-1 populations, at least initially, were comparable between the two hosts. This is because early in the experiment, when there exists lower overall diversity, genetic diversity can be approximated to be proportional only to mutation input[42]. Second, we observed a significantly higher rate of diversity loss in MT-2 lines compared to MT-4 (P <

148 0.001, paired t-test; supplementary fig. S5), reflected by a greater number of minority mutations (0.01 ≤
149 frequency < 0.5) disappearing between each consecutive samples.

150 *Mutations Continue to Fix at Approximately Constant but Host-Specific Rates*

151 Next, we analyzed the temporal patterns of fixed mutations. We define mutations as "fixed" if they are
152 present at a frequency of 99% or more in the population of a given sample. In total, we identified 414
153 single-base substitutions and 2 short deletions that reached fixation in either of four lines at some point
154 during the experiment. The strong bias towards single-base substitutions over indels in the HIV-1
155 genome evolution is well-documented in both patient and cell culture studies, and is attributed to a
156 combination of mutational mechanisms and selective pressures[43–45].

157 Mutations were fixed at an approximately constant rate throughout the experiment in each
158 population (Fig. 2B). The ongoing near-linear fixation of mutations despite saturation of genetic diversity
159 resembles patterns of evolving HIV-1 populations in treatment-naïve patients[46,47]. The rate at which
160 mutations fix can be calculated as the slopes in Fig. 2B. Assuming a generation time of 1.2 days, the
161 fixation rates are 0.085 and 0.042 per generation for MT-2 and MT-4 lines, respectively. Therefore,
162 mutations fixed virtually twice as fast in MT-2 ($P < 0.001$).

163 To pinpoint the source of the extra fixed mutations in MT-2 lines, we categorized them based on
164 their translational impact. Fixed mutations were most densely distributed in non-coding regions
165 (supplementary fig. S6); but, the ratio of coding to non-coding fixed mutations for MT-2 and MT-4 lines
166 was not significantly different from each other. In protein coding regions, however, we observed
167 significantly larger ratio of synonymous to nonsynonymous fixed mutation in MT-2 lines. While the
168 number of synonymous fixed mutations per synonymous site was significantly higher than that of
169 nonsynonymous in every line ($P < 0.001$, paired t-test; supplementary fig. S7), the effect size of this
170 difference was considerably larger in MT-2 compared to MT-4 lines (Cohen's d = 1.18 vs. 0.54,
171 respectively). These results suggest that the excess of fixed mutations in MT-2 lines predominantly came
172 from synonymous mutations.

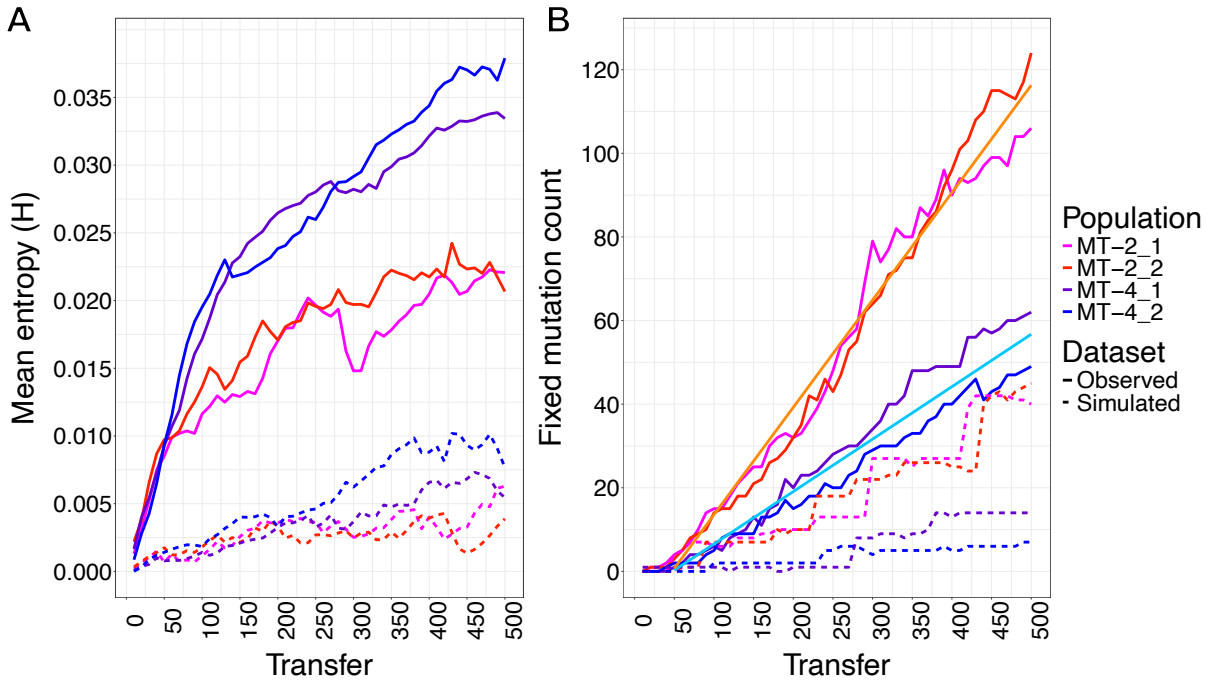


Fig. 2. Genomic patterns of diversity and divergence. (**A**) Genetic diversity over time. Population-level genetic diversity was measured per nucleotide by the Shannon's index and averaged over all the positions. The Shannon entropy per site quantifies uncertainty in the base composition of a randomly chosen genome. For DNA sequences, it ranges from H = 0 meaning the base nucleotide of a site is identical across genomes (only one nucleotide present) to H = 2 bits meaning all 4 nucleotides are equally frequent (P = 0.25 each) at a given site. The mean diversity for a sample was then calculated by averaging Shannon indices over the length of the genome. Diversity of all lines increase similarly over the first 60 transfers, but afterwards the rate slows down earlier for MT-2 lines. Moreover, diversity levels for MT-2 lines fluctuate more than MT-4 lines, indicating less stability in the MT-2 populations. Shannon diversity of simulated neutrally evolving populations reflected the observed patterns (dashed lines, refer to Methods). **B**) Count of fixed mutations over time. Mutations continued to fix (frequency ≥ 0.99) at an approximately constant rate even towards the end of experiment. There were no significant deviations from linearity (ANOVA, $P > 0.05$). The four evolution lines harbored 106, 124, 62, and 49 fixed mutations at transfer 500, respectively. The extrapolated slopes of 0.256 and 0.125 (fixed mutations per transfer) for MT-2 and MT-4 lines are shown in orange and light blue, respectively. Simulation of neutral sequence evolution (dashed lines, refer to Methods) suggests that 37% (~43 mutations) in MT-2 and 19% (~11 mutations) in MT-4 of the observed fixed mutations are expected to arise by neutral evolution alone.

192 *High Parallelism Observed Among Fixed Mutations*

193 Parallel evolution is the independent evolution of identical genetic changes and can arise when the
194 same adaptive solutions are repeatedly favored by selection[48]. Therefore, parallelism at the nucleotide
195 level is often used to identify potential targets of selection and gauge its strength[26,33]. We measured
196 parallelism among fixed mutations across evolutionary lines. As all our evolutionary lines were initiated
197 from a genetically homogenous ancestral population, the significance of observing parallel fixed
198 mutations is that these genetic changes had to first independently arise by mutation in each evolution
199 line and then be driven to fixation by selection.

200 Fig. 3A shows a Venn diagram of fixed mutations present at transfer 500 and highlights
201 considerable parallelism among different lines. Overall, 51 out of 274 distinct fixed mutations at transfer
202 500 (18.6%) evolved and fixed independently in at least two evolution lines, and 13 mutations (4.7%) in
203 at least three lines. Notably, three fixed mutations emerged and fixed among all four lines. Two of these
204 occurred in the 5′ untranslated region (5′UTR) and 5' long terminal repeats leading sequence (5′LTRLS)
205 of the HIV-1 genome (G566A and G678A). The third mutation was a nonsynonymous mutation in *env*
206 (A7854G or Asp545Gly). This Asp-to-Gly substitution is anticipated to have a low biochemical distance
207 (Grantham score of 56[49]), suggesting it is unlikely to disrupt *env* structure or function. This mutation
208 represents a reversion to the so-called HIV-1 "consensus of consensus" sequence compiled from all
209 HIV-1 genomes in the Los Alamos database.

210 Overall, we observed high levels of genetic parallelism across all lines, but to varying degrees.
211 Fig. 3B summarizes parallelism among fixed mutations between each line. At transfer 500, 19.4% of
212 fixed mutations in MT-4 lines were shared between its two replicates, while this number was 6.5% for
213 MT-2 replicates. Of note, the average level of parallelism between replicates of different environments
214 was 8.55%.

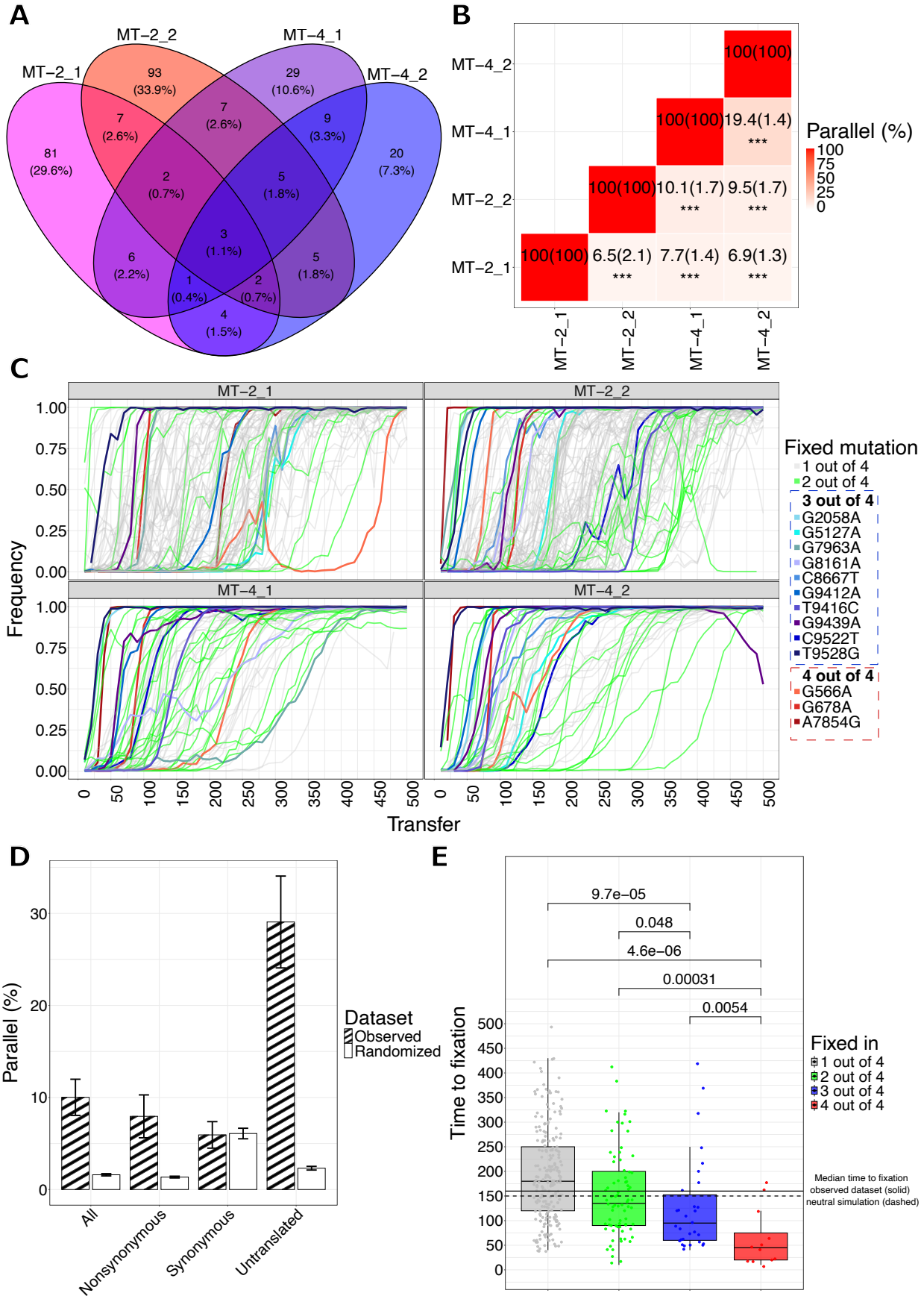
215 *Observed Parallelism Exceeds the Level Expected Under Neutral Evolution*

216 We carried out permutation tests to determine to what extent the observed levels of parallelism of fixed
217 mutations deviate from the expectation under neutral evolution. The permutation tests entailed
218 generating expected fixations by randomly drawing from the pool of all mutations (with the same
219 reference and alternative alleles) observed above 0.05 frequency in each evolution line at some point
220 throughout the experiment (refer to Methods). By this approach, we accounted for the potential

221 contributions of mutation bias and purifying selection to parallelism. The results of permutation tests
222 (Fig. 3B, numbers in brackets) confirmed that in all lines the observed levels of parallelism were
223 significantly greater than expected under neutral evolution.

224 Additionally, randomization tests accounting for the cell line indicated that parallelism observed
225 between MT-4 replicates was significantly larger than expected if the fixed mutations were to be
226 randomly redistributed between evolution lines, while the same effect was not seen for MT-2 replicates
227 (supplementary fig. S8). A contributing factor to the reduced parallelism observed in MT-2 relative to MT-
228 4 lines is genetic hitchhiking[50]. In line with signatures of genetic hitchhiking, the frequency trajectories
229 of MT-2 fixed mutations in Fig. 3C appeared to be more synchronized than MT-4 trajectories. More
230 prevalent hitchhiking in MT-2 lines can be the result of stronger linkage. Indeed, direct comparison of
231 linkage disequilibrium confirmed this pattern: MT-2 lines exhibited significantly stronger linkage than MT-
232 4 lines (supplementary fig. S9; paired *t*-test, $P < 0.001$).

233 We observed two conspicuous examples of genetic hitchhiking in our experiment. In MT-2_1,
234 between transfers 270 and 310, a set of 22 mutations doubled in frequency and reached fixation by
235 transfer 500. Of these mutations, 7 were synonymous: 5 unique to a single line and 2 fixed in two lines.
236 In a second case in MT-2_2, between transfers 160 and 200, a set of 25 mutations likewise doubled in
237 frequency and reached fixation by transfer 500. This set included 12 synonymous mutations: 11 unique
238 to a single line and 1 fixed in two lines. These cases highlight how multiple, probably neutral synonymous
239 mutations can rise in frequency synchronously and fix within individual MT-2 lines yet show little
240 convergence across lines. These observations provide an explanation for our finding above that
241 synonymous mutations account for most of the fixations in MT-2 lines.


A
MT−2_1
MT−2_2
MT−4_1
MT−4_2
81 (29.6%)
93 (33.9%)
29 (10.6%)
20 (7.3%)
7 (2.6%)
7 (2.6%)
9 (3.3%)
2 (0.7%)
5 (1.8%)
3 (1.1%)
6 (2.2%)
5 (1.8%)
1 (0.4%)
2 (0.7%)
4 (1.5%)
B
100(100)
100(100) 19.4(1.4) ***
100(100) 10.1(1.7) *** 9.5(1.7) ***
100(100) 6.5(2.1) *** 7.7(1.4) *** 6.9(1.3) ***
Parallel (%)
100
75
50
25
0
C
Frequency
Transfer
Fixed mutation
1 out of 4
2 out of 4
3 out of 4
G2058A
G5127A
G7963A
G8161A
C8667T
G9412A
T9416C
G9439A
C9522T
T9528G
4 out of 4
G566A
G678A
A7854G
D
Parallel (%)
Dataset
Observed
Randomized
All
Nonsynonymous
Synonymous
Untranslated
E
Time to fixation
9.7e−05
0.048
4.6e−06
0.00031
0.0054
Fixed in
1 out of 4
2 out of 4
3 out of 4
4 out of 4
Median time to fixation
observed dataset (solid)
neutral simulation (dashed)

242

243 **Fig. 3. Abundance and characteristics of parallel fixed mutations.** (**A**) Venn diagram depicting
244 number of shared and unique fixed mutations at transfer 500. An annotated list of these fixed mutations
245 can be viewed via the data table in LTEEviz. Overall, out of 274 distinct fixed mutations present at
246 passage 500, 223 (81.4%) are found in only a single evolution line. (**B**) Summary heatmap showing
247 percentage of parallel fixed mutations between each two replicates. Numbers in parentheses contain
248 expected level of parallelism computed by permutation tests. In all comparisons, observed parallelism
249 was significantly higher than expected. (**C**) Frequency trajectories of fixed mutations. Mutations fixed in
250 one and two populations are shown by grey and green, respectively. Mutations fixed in three and four
251 populations are shown individually by shades of blue and red, respectively. (**D**) Percentage of parallel
252 fixed mutation among each two lines in observed and randomized datasets per translational impact
253 group. Synonymous fixed mutations were not significantly different than expected, while fixed mutations
254 in nonsynonymous and non-coding regions were significantly more parallel in observed dataset
255 compared to randomized (Cohen's d = 1.6 vs. 3.1, respectively). Error bars represent the mean ±
256 standard error. (**E**) Time-to-fixation of fixed mutations based on their number of lines they were fixed in.
257 Time-to-fixation was defined as the number of transfers it took a mutation to appear above 0.01
258 frequency and (continuously) reach fixation by surpassing 0.99 frequency. The solid and dashed lines
259 depict the median time-to-fixation from the observed dataset (160) and neutral sequence simulation
260 (150), respectively.

261 *Evidence for Higher Fitness of Parallel Fixed Mutations*

262 In addition to the evidence for excess parallelism from permutation tests, specific characteristics of these
263 mutations further support the key role of selection in driving them to fixation. Firstly, synonymous
264 mutations are underrepresented among parallel fixed mutations. Only 6% of all parallel fixed mutations
265 were synonymous, while around 21% of all possible mutations in HIV-1 genome would lead to a
266 synonymous change. In fact, the level of parallelism in synonymous mutations was not significantly
267 different than what we expect under neutral evolution (Fig. 3D).

268 Secondly, the frequency trajectories of parallel fixed mutations appeared to rise more sharply
269 depending on how many times they emerged as fixed across different lines (Fig. 3C). To quantify their
270 fixation kinetics, we measured the duration from their appearance to their fixation (time-to-fixation).
271 Parallel fixed mutations exhibited significantly shorter time-to-fixation ($P < 0.05$, Wilcoxon rank-sum test)

272 with a clear trend: the time to fixation decreased with the number of evolution lines in which the mutation
273 occurred (Fig. 3E). For instance, the median time-to-fixation for mutations fixed in all four lines was
274 approximately 45 transfers, compared to 200 transfers for line-specific mutations (those fixed only in
275 one line). The monotonic decrease in time-to-fixation with increasing parallelism strongly supports the
276 role of selection in driving parallel mutations to fixation. As the time to fixation of highly-parallel fixed
277 mutations (those fixed in three or four lines with total of 13 distinct mutations) is significantly lower than
278 median time to fixation of neutral sequence simulation ($P < 0.01$, Wilcoxon rank-sum test), we consider
279 them as "putatively adaptive" and focus on their dynamics in the next section.

280 *Diminishing-Returns, Not Sign Epistasis, Define Interactions Between Adaptive Mutations*

281 To obtain a complete picture of the evolutionary dynamics in our long-term experiment, we investigated
282 epistatic interaction among adaptive mutations. First, we focus on the most extreme form of epistasis:
283 sign epistasis which reverses the fitness effect of a mutation from positive to negative, or vice versa[51,52].
284 To test whether sign epistasis had been involved in constraining emergence of adaptive mutations, we
285 inspected the chronological order of their fixation (Fig. 4A). We found that the three mutations that fixed
286 all four lines did so in three different orders across the lines. Additionally, five out of 10 mutations that
287 were fixed in same three lines did not follow any pattern in their order of fixation. A distinct order of
288 fixation per line among adaptive mutations suggests that these mutations were beneficial in different
289 genetic backgrounds.

290 We next tested whether the magnitude of fitness effects of adaptive mutations was modulated
291 by the genetic background, without changing their sign. To quantify the mutational effects, we used the
292 "initial relative fitness" metric proposed by Lang *et al.* (2011)[53]. The premise of this measurement is that
293 the initial frequency increase of a mutation should be proportional to the selective advantage it provides
294 in its genetic context relative to the population average. Consistent with previous findings,[25] logistic
295 regression modeling confirmed that probability of fixation of mutations was partly explained by their initial
296 relative fitness (supplementary fig. S10).

297 To test the temporal trends of fitness effects of adaptive mutations, we fitted linear regression
298 models for initial relative fitness of adaptive mutation as a function of time for each evolution line
299 separately (Fig. 4B). All four models confirmed a significant (adj.$P < 0.05$) negative log-linear relationship

300 between initial relative fitness and time (represented as the relative order of fixation of adaptive
301 mutations relative to each other within a single line).

302 Based on models' slope estimates, each adaptive mutation was associated with 27.8% (MT-2)
303 and 15.9% (MT-4) lower initial relative fitness compared to the previous adaptive mutation in the same
304 line. In other words, 90% of projected fitness gain by these adaptive mutations was achieved within 260
305 and 310 transfers (~720 and ~930 generations) in MT-2 and MT-4 populations, respectively
306 (supplementary fig. S11). Additionally, we found that the initial relative fitness of the same adaptive
307 mutation was 1.89 times larger (median) in the evolution line in which it went to fixation earlier ($P < 0.05$,
308 Wilcoxon rank-sum test, supplementary fig. S12). These results highlight the presence of strong and
309 pervasive diminishing-returns epistasis in our HIV-1 populations, causing adaptive mutations to confer
310 consistently smaller fitness gains in fitter backgrounds.

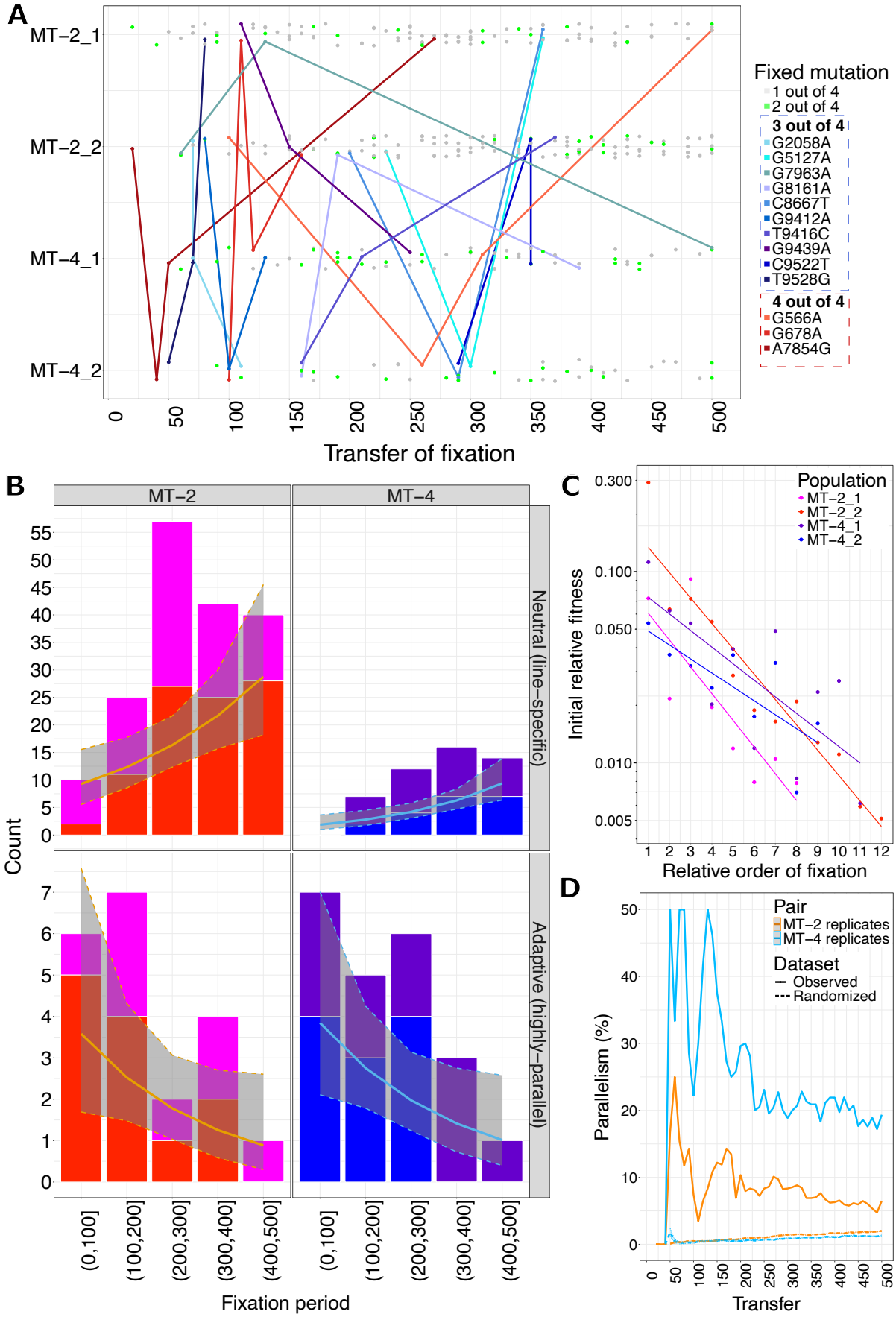
311 Moreover, accumulation of adaptive mutations slowed down considerably over the course of the
312 experiment (Fig. 4C). We utilized generalized linear mixed-effects models to quantify the downtrend in
313 counts of adaptive mutations and contrast it to that of neutral mutations. Our models confirmed that the
314 count of adaptive (highly-parallel) mutations significantly decreased over time by multiplicative factors
315 of 0.57 and 0.59 per 100 transfers in MT-2 and MT-4 populations, respectively. This pattern likely results
316 from a limited supply of beneficial mutations with large effects and their progressively shrinking beneficial
317 effects due to the influence of diminishing-returns epistasis. In contrast, line-specific fixed mutations
318 increased significantly over time, rising by multiplicative factors of 1.56 and 1.9 per 100 transfers in MT-
319 2 and MT-4, respectively. Because these line-specific mutations are likely neutral and fixed through
320 genetic drift and hitchhiking, this pattern reflects the ongoing activity of stochastic processes. Overall,
321 our models strongly support an opposite temporal trend in fixation rate of adaptive (highly-parallel) and
322 neutral (line-specific) fixed mutations in both hosts ($P < 0.01$, refer to supplementary fig. S13 for model
323 results and performance).

324 *Parallelism Decreases as Populations Continue to Evolve*

325 Bertels *et al.* (2019) also noted diminishing fitness effects among majority mutations (frequency ≥ 0.5)
326 by passage 90 but did not report a corresponding reduction in parallelism. We hypothesized that, over
327 longer timescales, a decrease in parallelism might be observable. This expectation is grounded in not

328 only the declining supply of adaptive mutations with progressively smaller fitness gains as we showed,
329 but also in the constant activity of drift fixing neutral mutations.

330 To test this hypothesis, we quantified the proportion of parallel fixed mutations relative to all
331 fixed mutations between replicates of each host over time (Fig. 4D). The proportion of parallel fixed
332 mutations among MT-2 evolution lines peaked at 25% at transfer 60 (2 out of 8 mutations) and dropped
333 to 6.5% by transfer 500 (14 out of 216 mutations). Similarly for MT-4 evolution lines, parallelism peaked
334 at 50% at transfer 130 (6 out of 12 mutations) and dropped to 19.4% by transfer 500 (18 out of 93
335 mutations). The time trends of proportion of parallel fixed mutations was congruent to that of the rate of
336 nonsynonymous to synonymous fixed mutation per site (supplementary fig. S14). By contrast,
337 permutation tests indicated that, under neutral evolution, parallelism is expected to gradually increase
338 over time (Fig. 4D). This pattern emerges as random processes continue to fix mutations across a finite
339 set of (nearly) neutral genomic loci.


A
MT−2_1
MT−2_2
MT−4_1
MT−4_2
0
50
100
150
200
250
300
350
400
450
500
Transfer of fixation
Fixed mutation
1 out of 4
2 out of 4
3 out of 4
G2058A
G5127A
G7963A
G8161A
C8667T
G9412A
T9416C
G9439A
C9522T
T9528G
4 out of 4
G566A
G678A
A7854G
B
MT−2
MT−4
Neutral (line−specific)
Adaptive (highly−parallel)
Count
(0,100]
(100,200]
(200,300]
(300,400]
(400,500]
Fixation period
C
Population
MT−2_1
MT−2_2
MT−4_1
MT−4_2
Initial relative fitness
0.300
0.100
0.050
0.010
0.005
Relative order of fixation
D
Pair
MT−2 replicates
MT−4 replicates
Dataset
Observed
Randomized
Parallelism (%)
Transfer

340

341 **Fig. 4. Interactions between parallel mutations and their temporal dynamics.** (**A**) Order of fixed
342 mutations based on the transfer at which they fixed. Mutations fixed in one and two populations are
343 depicted by grey and green points, respectively (lines for mutations fixed in two populations are omitted
344 for clarity). Mutations fixed in three and four populations are depicted case by case with points and
345 connecting lines in shades of blue and red, respectively. Out of 10 mutations that fixed in three lines,
346 five (G2058A, G8161A, G9412A, T9416C, C9522T) fixed in the same three lines (MT-2_2, MT-4_1, MT-
347 4_2), but their order of fixation did not contain any pattern. (**B**) Counts of neutral (line-specific) and
348 adaptive (highly-parallel) fixed mutations per their fixation period aggregated for replicates of MT-2 and
349 MT-4. Each bin represents 100 transfers. Notably, the number of neutral (line-specific) mutations is
350 markedly larger in MT-2 lines. (**C**) Initial relative fitness vs. relative order of fixation (time). Each point
351 represents an adaptive mutation colored by the respective line they occurred in. The x-axis represents
352 the order at which each adaptive mutation was fixed relative to other adaptive mutations in the same
353 line. Therefore, x-axis represents relative time specific to each line. All fitted regression lines are
354 significant after multiple testing correction. The magnitude of decline tended to be greater in MT-2 than
355 in MT-4 lines (average slope estimate of -0.471 vs. -0.251), but the difference was not statistically
356 significant between the two host environments. (**D**) Parallelism among fixed mutations between
357 replicates of MT-2 and MT-4. The neutral sequence simulation (dashed lines) estimates increasing
358 expected parallelism among fixed mutations over time (ribbons indicate lower and upper 95% CI).
359 Conversely, the observed level of parallelism is generally decreasing since transfer 60 and 130 in MT-2
360 and MT-4 replicates, respectively. The decreasing parallelism indicates positive selection during the
361 beginning of the experiment was stronger.

362 *A Selective Sweep Occurs After Deceleration of Adaptation in MT-2_1*

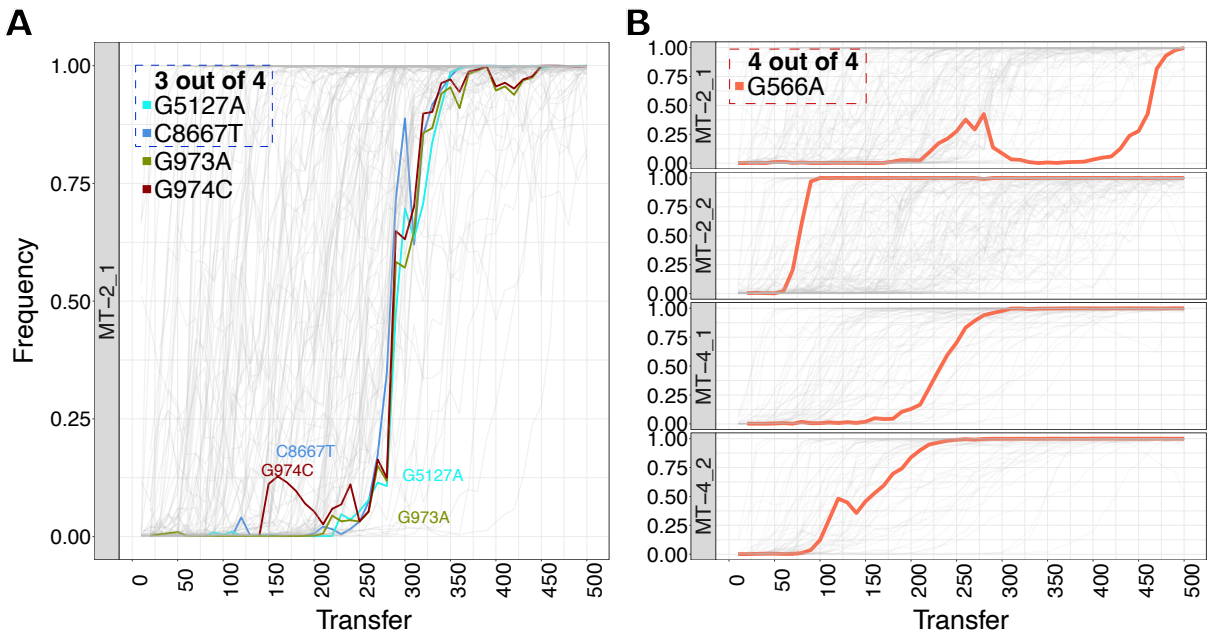
363 Patterns of genomic diversity and divergence support the occurrence of a (hard) selective sweep in MT-
364 2_1 from transfer 270 to 310, after the adaptation had decelerated substantially in this evolution line.
365 First, during this period, the rate of divergence in MT-2_1 was increased significantly as manifested in
366 the reconstructed phylogeny (supplementary fig. S15). Second, this period coincided with a 20% drop
367 in diversity, followed by a rebound to equilibrium levels (Fig. 2A). The diversity reduction is one of the
368 main genetic hallmarks of (hard) sweeps[54,55], which is caused by a single genotype outcompeting every
369 other genotype and purging polymorphism surrounding the site(s) undergoing selection.

370 Identifying the driver of this selective sweep with the available data is challenging, but several
371 cases among the set of 22 mutations that doubled in frequency during this period and reached fixation
372 by transfer 500 are worth noting. The first case is the emergence of a rare amino acid change in *gag*.
373 Two mutations whose frequencies rose sharply and in a synchronized manner during this period
374 occurred at adjacent positions 973, and 974 within the *gag* gene (Fig. 5A). These two mutations are
375 nonsynonymous and affect the same codon (codon 62 of the 500-residue *gag* MA protein), with each
376 mutation leading to a different amino acid change if it occurs independently. Frequency trajectories of
377 these mutations indicate that G974C appeared first, resulting in Gly62Ala amino acid change, followed
378 by the emergence G973A. Based on inspection of raw sequencing reads, G973A occurred in the
379 background of the G974C, leading to a subsequent Ala62Thr amino acid change at the same position
380 of the amino acid. Of note, there was no single nucleotide change in that codon that could have resulted
381 in direct Gly → Thr amino acid change at position 62 of *gag* MA protein. Moreover, both G973A and
382 G974C were not observed above frequency of 0.05 in any other line than MT-2_1. This observation
383 suggests that each of these mutations separately could have been deleterious for the virus, while their
384 co-emergence reversed their mutational effect (reciprocal sign epistasis) from deleterious to beneficial
385 (or neutral), and thereby facilitated their fixation in MT-2_1.

386 Additionally, two adaptive mutations (G5127A and C8667T) also rose synchronously with the
387 sweep. Both mutations were nonsynonymous: G5127A hit *vif*, causing a Met29Ile amino acid change;
388 while C8667T hit *env*, resulting in Thr816Ile. Whether any of these mutations were the driver of this
389 sweep or not cannot be inferred from our data, but their presence in the outcompeting genotype could
390 provide further fitness advantage over the existing genotypes in the population.

391 Independent evidence in support of the fitness benefit of this sweeping genotype comes from
392 its clonal interference with another genotype in MT-2_1, which carries the beneficial mutation G566A
393 (Fig. 5B). G566A was fixed in all four lines with average time-to-fixation of 125 transfers compared to
394 the overall average of 170 transfers, pointing to its potential benefit for the fitness of the virus. Its
395 frequency paths to fixation were straightforward in all lines, except in MT-2_1. In MT-2_1, G566A's
396 frequency trajectory was interrupted by the occurrence of the selective sweep: it appeared at transfer
397 180 and rose to around 0.44 frequency by transfer 280, when it was overtaken by the sweeping genotype
398 and disappeared from the population. Later in the experiment, it reappeared by an independent de-novo

399 mutation event and reached fixation by transfer 500. This observation emphasizes the adaptiveness of
400 the sweep and the competition that exist between sub-populations[25].



401

402 **Fig. 5. Selective sweep and clonal interference in MT-2_1.** (**A**) Frequency trajectories of potential
403 drivers of the selective sweep. Although we lack haplotype information linking these sites together, their
404 tightly correlated trajectories, together with a 20% drop in global diversity despite ongoing
405 recombination, suggest that they could have resided within the same sweeping genotype. (**B**) An
406 example of clonal interference between the sweeping genotype and another genotype with a putatively
407 beneficial mutation in MT-2_1. G566A emerged and rose to fixation continuously and through a
408 straightforward trajectory in all evolution lines, but in MT-2_1. In MT-2_1, its frequency trajectory was
409 interrupted by the selective sweep. After being eliminated from the population following the sweep,
410 G566A re-emerged at transfer 410 and reached fixation by transfer 500 in MT-2_1 population.

411

412 ## Discussion

413 In this study, we investigated the adaptive dynamics of HIV-1 based on its genome evolution in a long-
414 term evolution experiment. We observed excess parallel evolution of fixed mutations and provided three
415 independent lines of evidence to establish their adaptive nature. First, these mutations were highly
416 unlikely to be parallel under neutral evolution based on randomization test results. Second, they were

417 significantly underrepresented among synonymous mutations. Third, their time to fixation was
418 significantly lower than that of line-specific fixed mutations or fixed mutations in the neutral evolution
419 simulation.

420 In the absence of intentionally imposed selective pressures in our study (e.g., immune response
421 or antiretroviral drugs), we expect acclimatization to distinct cell line hosts and in vitro growth conditions
422 to represent the primary source of selective pressure. By examining the prevalence and epistatic
423 interactions among the adaptive mutations, we showed that the rate of adaptation decelerated
424 substantially over the course of the experiment: the fixation rate of adaptive mutations almost halved
425 within the first 300 transfers, while their conferred fitness gain decreased by 27% (MT-2) and 18% (MT-
426 4) per every added adaptive mutation in their genetic background. This deceleration was also reflected
427 in infectivity data obtained via titration assays: in all evolutionary lines, we detected an initial phase of
428 increasing infectivity up to transfer 150 in MT-2 and transfer 300 in MT-4 populations, after which
429 infectivity levels stabilized.

430 Previous studies documented the deceleration of adaptation of evolving microbial populations
431 in constant environments for a diverse set of microorganisms such as bacteria[26], phage[56], and yeast[3],
432 indicating that this phenomenon transcends the specifics of the biological system[14]. Here, we highlight
433 that the timing and scale of such deceleration can vary greatly based on the molecular and demographic
434 properties of the model organism[57,58]. In *E. coli*, for instance, Tenaillon *et al.* (2016)[26] reported that "the
435 proportion of observed mutations that were beneficial declined over time but remained substantial even
436 after 50,000 generations". By contrast, our analysis of HIV-1 genome evolution indicates that a
437 substantial decline in adaptation was reached within the course of our experiment, which corresponds
438 to 1,500 HIV-1 generations. To quantitatively compare the two experiments, we relied on the
439 measurement of the rate of nonsynonymous to synonymous mutations reported in Tenaillon *et al.*
440 (2016). In *E. coli* populations, this rate dropped to two at generation 50,000, while in our HIV-1
441 populations the rate of two was reached by transfer 70 (generation ~210) and 230 (generation ~690) in
442 MT-2 and MT-4, respectively.

443 Several factors could underlie earlier deceleration of adaptation in our HIV-1 populations
444 compared to that of *E. coli* populations. Most important among those is HIV-1's enhanced ability to
445 diversify due to higher mutation rate and possibility of recombination compared to *E. coli*. Mutations are

446 the fuel for selection; the faster adaptive mutations appear, the earlier they will get fixed in the population
447 by natural selection. However, despite the mutation rate estimates of HIV-1 being several orders of
448 magnitude higher (~2 × 10^{-5} mutations per nucleotide per replication[29] vs. 8.9 × 10^{-11} per base-pair per
449 generation in non-mutator lineages of *E. coli*[59]), the population mutation supply ($\mu \times N_e$) was only
450 around two orders of magnitude larger for HIV-1 populations (3.17 × 10^{-2} vs. 8.9 × 10^{-4}). To what extent
451 does this difference in mutational supply explain the timescale of the deceleration of adaptation will need
452 to be answered by detailed population genetic simulation studies in the future.

453 Recombination can also increase the rate of appearance of new genotypes through random
454 shuffling of the genetic material between different haplotypes[60]. In doing so, it alleviates the clonal
455 competition between sub-populations by increasing the efficacy of selection (the Fisher-Muller effect[61–
456 63]). While the reproduction of *E. coli* strain was strictly clonal (i.e., no horizontal gene transfer[64]), HIV-1
457 can recombine through template switching[65], resulting in faster adaptation over a wide range of
458 conditions as shown previously[66]. The occurrence of recombination in our evolving HIV-1 populations
459 was evident from the fast decay in linkage disequilibrium of mutations within the span of read pairs.

460 Notwithstanding the substantial decline in rate of adaptation, we observed that all populations
461 continued to diverge from the ancestor at a relatively constant rate even towards the end of the
462 experiment. Such prolonged period of linear accumulation of fixed mutations has been observed in
463 microbial populations in similar studies[25,67]. This continuing accumulation can be, in part, attributed to
464 the steady fixation of (nearly) neutral mutations via genetic drift[26]. Our simulations of neutral sequence
465 evolution estimated that 37% (~43 mutations) in MT-2 and 19% (~11 mutations) in MT-4 lines could have
466 been fixed by drift alone. The sources of drift in our experiment are periodic bottlenecking, variation in
467 *R0* among individual infections, and founder effects due to finite population sizes. But resolving the
468 discrepancy between the linear accumulation of fixations on the one hand, and the deceleration of
469 adaptation, on the other, will require further investigation.

470 Two limitations of our study are worth noting. First, although we substantiated the role of
471 selection in giving rise to parallelism among fixed mutations across evolutionary lines, we did not
472 disentangle the potential contributions of biased mutation rates to the extent of observed parallel
473 evolution[68–71]. Nonetheless, we believe the undesired influence of mutational bias should be mitigated
474 by focusing on parallelism at the level of fixed mutations. The second limitation, inherent to the use of

475 short-read pooled sequencing data, is the lack of haplotype information. Available computational tools
476 failed in reconstructing haplotypes, restricting us from thoroughly examining clonal dynamics within
477 populations and interactions between mutations. As a workaround, we resorted to identifying and
478 characterizing instances of such phenomena using frequency trajectories and the partial linkage
479 information that was available within the short sequencing read pairs. That said, the availability of frozen
480 samples offers a promising avenue for future studies to obtain single-read coverage of the entire HIV-1
481 genome using long-read sequencing technologies[72,73].

482 High-throughput sequencing datasets such as those generated in this study demand extensive
483 preprocessing such as quality filtering, read mapping, and downstream data handling before they can
484 effectively inform specific biological questions. As a result, although public deposition of raw sequencing
485 data is essential, these files alone often have limited practical value for secondary analyses. To enhance
486 accessibility, ensure transparency, and promote broader reuse, we created an interactive web-based
487 platform (LTEEviz) that enables users to examine and visualize the dataset dynamically. The platform
488 also offers straightforward access to the curated dataset, lowering the technical barrier for reanalysis.
489 We anticipate that this resource will support further investigation and enable new insights beyond the
490 scope of the present study.

491 In summary, our results revealed a swift and substantial decline in the rate of adaptation in our
492 HIV-1 populations. We propose fast diversification as a key driver of such rapid adaptation in HIV-1
493 compared to that of other microbial populations in similar studies. Future controlled laboratory
494 experiments or computer simulations are needed to model how diversification rates, along with other
495 potentially relevant biological determinants, influence the timing and extent of deceleration in
496 adaptation of microbial populations.

497

498 # Material and Methods

499 *Passaging of HIV-1*

500 The detailed description of HIV-1 passaging in this LTEE study can be found in our previous
501 publication[32]. In summary (refer to Fig. 1A for a graphical overview), the ancestral virus HIV-1_{NL4-3} strain
502 (generated and characterized as previously indicated[74]) was passaged in four independent evolutionary

503 lines in two biological replicates of human leukemia T-cell cell lines[75], MT-2 and MT-4. The HIV-1 full-
504 length plasmid pNL4-3 and the two cell lines were obtained through the AIDS Research and Reference
505 Reagent Program, Division of AIDS, NIAID, NIH from Dr. Malcolm Martin[76] and Dr. Douglas Richman,
506 respectively.

507 At every 3rd or 4th day, 2 µl of infected cell suspension was transferred to a new flask containing
508 4×10^5 uninfected cells. At every 10th transfer, cell-free supernatant of infected cell suspension was
509 obtained through centrifugation and stored at -80°C freezer. Of note, every few months a fresh batch of
510 cells were thawed and used for the continuation of the experiment to avoid potential confounder effects
511 associated with co-evolution of cell lines with viral lines.

512 *Sequencing of HIV-1 Genome*

513 Full-length genome of the virus stock HIV-1_{NL4-3} (ancestor) and every 10th transfer until transfer of 500
514 (4 × 50 = 200, in total) were sequenced using the Illumina MiSeq next-generation sequencing platform
515 (Illumina, San Diego, California, United States of America) as previously described[74]. This Briefly, HIV-
516 1 RNA was isolated from 150 µl virus stock HIV-1_{NL4-3} or cell-free supernatant. Five overlapping
517 amplicons were generated by reverse transcription polymerase chain reaction covering the full genome
518 of HIV-1 per sample. The 5 amplicons per sample were pooled, and libraries were prepared with the
519 Nextera® XT DNA Sample Preparation Kit (Illumina) according to the manufacturer's description. Next-
520 generation sequencing was performed using Illumina's MiSeq Sequencer, generating paired-end reads
521 of 2 × 250 bp length (v2 kit). To minimize the risk of cross contamination, samples from each replicate
522 line were processed separately.

523 *NGS Data Processing*

524 All the steps of processing raw sequencing reads were implemented and performed via a Snakemake
525 pipeline (supplementary fig. S16 data processing workflow; supplementary fig. S1 pipeline's directed
526 acyclic graph). First, using FASTQC and MULTIQC tools, we examined the quality of raw sequencing
527 reads. Sequencing samples that did not meet quality requirements were re-sequenced. Next, using
528 Trimmomatic we trimmed base calls with low quality (Phred score ≤20, corresponding to 0.01 error
529 probability) and removed short reads (length of ≤ 36 bases).

530 The trimmed reads were then mapped against the ancestral HIV-1_{NL4-3} consensus sequence
531 using BWA-MEM with default settings. Reads with mapping quality ≤ 35 (corresponding to error
532 probability of 0.00032) were discarded. The average sequencing depth each of the 5 amplicons were
533 calculated and if they fell below 1,000, the sequencing was repeated for that amplicon. The R-to-R viral
534 genome of HIV-1 for all samples had at least a coverage of 200 quality reads, resulting in full-length
535 sequencing of genome (for coverage summaries refer to supplementary fig. S17).

536 To call the variants, we used in-house python scripts (99% similarity with commonly used tools
537 such as smalt-align and mpileup). The genome coordinates are based on NL4-3 plasmid (NCBI
538 accession number: AF324493.2). A minor allele frequency (MAF) cutoff of 0.01 and sequencing depth
539 cutoff of 200 reads were applied to exclude potential erroneous sequencing calls. For downstream
540 analyses, mutation categories were defined based on the following variant frequency ranges:

Mutation Category	**Frequency**
Fixed	Freq. ≥ 0.99
Majority	Freq. ≥ 0.5
Minority	0.01 ≤ Freq. < 0.5

541

542 *Variant Annotation*

543 Genetic variants were functionally annotated using SnpEff[77] (v5.1), based on its official documentation.
544 Annotations included predicted effects on genes and proteins, such as amino acid changes. Genomic
545 feature list of the HIV-1_{NL4-3} strain was obtained from NCBI (accession no. AF324493.2) and was
546 manually updated based on the reconstructed ancestral sequence. The annotation includes nine
547 protein-coding genes and five distinct non-coding regions. Additionally, reversion sites were pinpointed
548 by gene-by-gene alignment of the virus stock's consensus sequence with the premade 2004 cross-
549 subtype consensus from the Los Alamos HIV Sequence Database. A mutation was classified as a
550 reversion when, in later transfers, the nucleotide at that position changed to match the database
551 consensus.

552 *$TCID_{50}$ Measurement*

553 We performed titration assay to determine the 50% tissue culture infectious dose ($TCID_{50}$) of evolving
554 HIV-1 populations. For the first 100 transfers, $TCID_{50}$ values were measured for every 10th transfer, and
555 after transfer 100, for every 100 transfers on their respective cell line. Briefly, 1×10^3 MT-2/4 cells/well
556 were plated in 100 µl of complete medium in two 96-well plates. Serial 1:2.5 dilutions of HIV-1 viral stock
557 were prepared as follows: dilution for row 1 was prepared by mixing 20 µl of cell-free supernatant virus
558 stock with 980 µl of medium (1:50 dilution), followed by a series of 1:3 or 1:4 dilutions (250 µl of the
559 previous dilution mixed with 500/750 µl of medium) until dilution row 11. Row 12 served as the negative
560 control with 500 µl of medium only.

561 Each viral dilution was added in quadruplicate (50 µl per well) to the pre-seeded MT-2/4 cells.
562 Plates were incubated at 37°C with 5% CO_2 for 7 days. On day 7 post-infection, viral replication was
563 assessed using microscopic analysis, identifying the presence of syncytia formation and extensive cell
564 debris as indicators of HIV-1 infection. The $TCID_{50}$ values were calculated using the Reed and Muench
565 method[78], modified by the Spearman-Kaerber formula[79]:

566 $$\log(\text{TCID}_{50}/\text{mL}) = X_0 - \frac{d}{2} + \mathrm{d} \times \frac{\sum(x_i)}{n} + \log(1/v)$$

567 where X_0 represents the initial dilution in the assay, d is the log dilution factor, x_i is the number of positive
568 wells per dilution i, n is the total number of wells, and v is the volume of virus inoculum per well.

569 The multiplicity of infection (MOI) for transfer t was then calcualted by the following formula
570 based on a Poisson approximation for the relationship between TCID50 and plaque forming units[80]:

571 $$\text{MOI(t)} = 0.69 \times \frac{\text{TCID}_{50}/\text{mL}(t) \times \text{transfer_volume}}{N}$$

572 where N is the number of seeded cells in each flask (400,000) and $\mathrm{transfer_volume}$ is the bottleneck
573 inoculum volume (2 µl).

574 Number of infectious units per bottleneck at transfer t ($IU_{bottleneck}(t)$) would then be simply
575 yielded from:

576 $$IU_{bottleneck}(t) = \mathrm{N} \times \text{MOI(t)}$$

577 Additionally, to assess cross-infectivity between the two environments, we performed titration
578 assay for viral stock of 100, 200, 300, 400, and 500 transfers of each evolving population on the opposite
579 cell line. The abovementioned formulas were then used to calculate $TCID_{50}$, MOI, and $IU_{bottleneck}(t)$.

580 *Estimating Effective Population Size*

581 We estimated the effective population size (N_e) under the Wright-Fisher model from the variance in
582 variant frequency change (σ_p^2) of (nearly) neutral alleles. We used synonymous variants as a simple
583 proxy for (nearly) neutral alleles. σ_p^2 of synonymous variants in the genome after *t* generations was
584 calculated as depicted by the equation below[81]:

585 $$\sigma_p^2 = p(1-p)[1-\left(1-\frac{1}{N_e}\right)^t]$$

586 where p is the initial variant frequency. If we consider *F* as the standardized variance[82]; where

587 $$F = \frac{\sigma_p^2}{p(1-p)}$$

588 then by approximating for large N_e (i.e., $\frac{1}{N_e} \approx 0$), we can derive

589 $$N_e \approx \frac{-t}{2\ln(1-F)}$$

590 *Genomic RNA Copy Number Quantification*

591 Quantification of HIV-1 genomic RNA copies in cell-free supernatants for every 10th transfer was
592 performed using quantitative PCR (qPCR) with SYBR Green detection on an ABI 7500 real-time PCR
593 system (Applied Biosystems). Reactions were carried out in duplicate in a 20 µl total reaction volume,
594 consisting of 15 µl qPCR master mix and 5 µl of sample (cDNA or standard dilution). The qPCR master
595 mix was prepared using 4 × real-time PCR PRE MIX diluted to 1× final concentration, containing 0.005
596 µM ROX, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, and 0.1× SYBR Green. Specific primers targeting the HIV-1
597 integrase region (INT:4452-4778) were added at a final concentration of 0.4 µM each, along with
598 JumpStart Taq DNA Polymerase (Sigma-Aldrich) at 1 U per reaction. The qPCR cycling conditions were
599 as follows: initial denaturation at 95°C for 3 min, followed by 50 cycles of denaturation at 94°C for 15
600 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. Fluorescence data were collected
601 at the 72°C extension step. A melting curve analysis was conducted immediately after amplification, with

602 a temperature ramp from 55°C to 95°C (1% increase per step), including fluorescence data collection.
603 Negative template controls (NTCs) were included using 5 µl of nuclease-free water in place of the cDNA
604 template. Standard curve quantification was performed using serial dilutions of wild-type (WT) HIV-1
605 RNA standards to determine absolute RNA copy numbers. The measurements were performed in
606 duplicates for each sample, and the average dilutions were reported.

607 *Simulation of Neutral Sequence Evolution*

608 To predict how many neutral mutations would arise in our experimental populations, we simulated the
609 evolution of individual HIV-1 genomes of length 9,171 (R-to-R) for 1,500 generations. We started our
610 simulation with a fixed number of 400 infection virions for both MT-2 and MT-4 simulations. Each
611 individual genome was assumed to produce 12 offspring genomes per generation (R_0 = 12), and every
612 newly produced viral genome was assumed to acquire a mutation with a probability of 2×10^{-5}. The
613 mutation target size of neutral positions was assumed to be proportional to the cumulative count of
614 mutated positions at transfer 500. The viral populations were then allowed to replicate exponentially for
615 three generations. Consistent with the experimental setup, we simulated a bottleneck every third
616 generation, by randomly selecting a certain number of individuals from the previous population. The size
617 of the bottleneck, that is, the number of infectious virions transferred at every transfer, was estimated
618 based on infectivity measures experimentally obtained by titration assay for 10th and 100th transfers
619 (refer to "$TCID_{50}$ Measurement" section above). To this end, we estimated $IU_{bottleneck}(t)$ for every
620 transfer by taking the logarithmic average of every 2 available datapoints:

621
$$IU_{bottleneck}(t) = \begin{cases} \dfrac{IU_{bottleneck}\left(10\left\lceil\frac{t}{10}\right\rceil\right) - IU_{bottleneck}\left(10\left\lfloor\frac{t}{10}\right\rfloor\right)}{\ln\left(IU_{bottleneck}\left(10\left\lceil\frac{t}{10}\right\rceil\right)\right) - \ln\left(IU_{bottleneck}\left(10\left\lfloor\frac{t}{10}\right\rfloor\right)\right)}; & where \quad 1 \le t \le 100 \\ \dfrac{IU_{bottleneck}\left(100\left\lceil\frac{t}{100}\right\rceil\right) - IU_{bottleneck}\left(100\left\lfloor\frac{t}{100}\right\rfloor\right)}{\ln\left(IU_{bottleneck}\left(100\left\lceil\frac{t}{100}\right\rceil\right)\right) - \ln\left(IU_{bottleneck}\left(100\left\lfloor\frac{t}{100}\right\rfloor\right)\right)}; & where \quad 101 \le t \le 500 \end{cases}$$

622 *Parallel Mutation Permutation Tests*

623 Neutral parallelism. To estimate the expected level of parallel evolution, we randomly redistributed the
624 fixed mutations observed in each evolutionary line across the genome in the following manner: for each
625 line, fixed mutations were placed at sites where amutation with the same reference and alternative
626 alleles had reached a frequency of at least 0.05 at some point during the experiment. With this approach,

627 we exclude the non-mutated positions (positions which their mutation could results in a lethal effect and
628 hence were not observed) to account for the confounding effects of purifying selection. After random
629 shuffling of fixed mutations over the pool of mutated positions, we measured the proportion of mutations
630 that are shared among each two lines. We then repeated the entire simulation 1,000 times for each pair
631 of lines.

632 However, this method may still underestimate neutral parallelism, since mutations that occurred
633 later in the experiment might not have had sufficient time to reach fixation. To address this, we subtracted
634 the median fixation time expected under genetic drift (estimated from neutral sequence simulations)
635 from the total experimental duration of 500 transfers and included only mutations that arose before that
636 adjusted time point. Accordingly, we applied median lag corrections of 140 and 185 transfers for the MT-
637 2 and MT-4 lines, respectively.

638 To determine the expected neutral parallelism for mutations per their translational impact, we
639 performed similar permutation tests. Roughly, 2%, 21%, and 77% of any mutation in the HIV-1 genome
640 would result in untranslated, synonymous, and nonsynonymous mutations, respectively. These
641 percentages were determined based on the consensus ancestral sequence.

642 Environment Contribution. Randomization tests were used to analyze the contribution of
643 environment to observed parallelism within and between each environment as previously described[36].
644 In short, we redistributed fixed mutations between different experimental lines, while keeping the count
645 of fixed mutations per each line and the number of occurrences of each fixed mutation unchanged.
646 Subsequently, randomized parallelism within and between each environment was calculated and
647 classified as outlined before. The dataset was randomized, and parallelism was classified in this manner
648 for 1,000 times, after which non-parametric significance tests were performed to compare the observed
649 values with the distribution of randomized values.

650 *Population Diversity Calculation*

651 We calculated and reported Shannon entropy[83] for population-level genetic diversity. The Shannon
652 index is a measure of diversity that quantifies the information content (entropy) in the sequencing data
653 of a population, representing the uncertainty in predicting the nucleotide base of a locus for a randomly
654 chosen genome. Shannon indices were obtained according to the following formula for each locus:

$$D_j(t) = -\sum_{i \in (A,C,G,T)} p_{ij} \log_2(p_{ij})$$

where $D_j(t)$ is entropy at locus j at transfer t, and p_{ij} is the proportion of each of the 4 possible nucleotides (A, C, G, T). We obtained the mean diversity $\overline{D_g}(t)$ over the HIV-1 genome with length L_g:

$$\overline{\mathrm{D_g}}(\mathrm{t}) = \frac{1}{L_g}\sum_{\mathrm{i}=1}^{\mathrm{L_g}} \mathrm{D_j}(\mathrm{t})$$

dN/dS Calculation

dN and dS were calculated and reported according to the following definitions:

- dN(*t*) = number of nonsynonymous mutations at transfer *t* / total number of nonsynonymous sites
- dS(*t*) = number of synonymous substitutions at transfer *t* / total number of synonymous sites

Time to fixation measurement

To estimate the time to fixation of mutations, we tracked their frequencies across passages for each experimental line. For each line, mutations that reached fixation (frequency ≥ 0.99 at transfer 500) were identified. The transfer at which a mutation first appeared was determined as the earliest passage with frequency ≥ 0.01, and the passage of fixation was recorded as the earliest passage with frequency ≥ 0.99. If the frequency after appearance dropped to below 0.01 for two consecutive transfers, the transfer of appearance was updated to the next transfer it reappeared. With this method, we computed the continuous time it took a mutation to appear and reach fixation.

Linkage disequilibrium

Linkage disequilibrium (LD) between single-base mutations was calculated using paired-end sequencing data with an in-house script. Mutations with a frequency ≥ 0.05 in each transfer were considered. For each pair of mutations separated by ≤ 400 bp, haplotypes were reconstructed from read pairs covering both positions. Reads and their mates were aligned to the reference genome, and nucleotides at the mutation positions were extracted to define haplotypes. Frequencies of the four

possible haplotypes (reference-reference (fab), reference-alternate (faB), alternate-reference (fAb), alternate-alternate (fAB)) were obtained. The disequilibrium coefficient (D) was calculated as follow:

$$D = fAB - pA * pB; \qquad where \qquad pA = fAB + fAb \ \& \ pB = fAB + faB$$

and the squared correlation coefficient (r^2) as a measure of LD was subsequently calculated as:

$$r^2 = \frac{(D^2)}{(pA \times (1 - pA) \times pB \times (1 - pB)}$$

Mutation pairs with no overlapping reads were assigned NA. LD calculations were performed in parallel across available cores to optimize computational speed.

Initial relative fitness calculation

To assess the relationship between fitness and fixation probability, we estimated initial relative fitness as described in Lang *et al.* (2011). In short, for each mutation, we identified t_1 and t_2, the first consecutive transfers where the frequency of the mutation was greater than 0.01 and 0.1, respectively. We then calculated initial relative fitness as:

$$Initial\ relative\ fitness = \frac{1}{t_2 - t_1}\left(\ln\frac{f(t_2)}{1 - f(t_2)} - ln\frac{f(t_1)}{1 - f(t_1)}\right)$$

where $f(t)$ is the frequency of the mutation at transfer t.

Website

The interactive web application for exploring and accessing the curated data was developed using the R Shiny package and is available at: https://lteeviz.shinyapps.io/lteeviz/

Use of Artificial Intelligence

We acknowledge the use of OpenAI's ChatGPT (GPT-4 and GPT-5) for language edits and Anthropic's Claude AI to assist with code debugging and syntax refinement. All text and code logic, design, and interpretation were performed and verified by the authors.

Code and data availability

All the code for data processing pipeline and downstream analyses can be found on GitHub at git@github.com:Al313/hiv1_genome_evolution.git. The Illumina sequencing data were deposited in the NCBI short read archive under the accession number PRJNA506879. The code for web application tool (LTEEviz) can be found on GitHub at git@github.com:Al313/lte-viz.git. The curated data can be obtained via LTEEviz.

Supplementary Material

Supplementary data are enclosed in a single PDF and will be made available online.

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C.L., K.N., R.C. and K.J.M. performed the experiments; K.J.M. and R.R.R. designed the experiments; A.M. analyzed the data; A.M. wrote the article; R.R.R. and K.J.M. provided feedback on the article; A.M. designed, coded, and implemented LTEEviz web application tool; L.S. contributed to the coding of LTEEviz. A.M. and L.S. were funded by European Union's Horizon 2020 research and innovation program, under the Marie Skłodowska-Curie Actions Innovative Training Networks grant no. 955974 (VIROINF). K.J.M. acknowledges the financial support of the Swiss National Science Foundation (grant no. SNF 204404). K.J.M. acknowledges the financial support of the 2021 fellowship program by Gilead Sciences (grant no. 13849).

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