Phylogenetic inference of changes in amino acid propensities with single-position resolution

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Abstract

Fitness conferred by the same allele may differ between genotypes, and these differences shape variation and evolution. Changes in amino acid propensities at protein sites over the course of evolution have been inferred from sequence alignments statistically, but the existing methods are data-intensive and aggregate multiple sites. Here, we develop an approach to detect individual amino acids that confer different fitness in different groups of species from combined sequence and phylogenetic data. Using the fact that the probability of a substitution to an amino acid depends on its fitness, our method looks for amino acids such that substitutions to them occur more frequently in one group of lineages than in another. We validate our method using simulated evolution of a protein site under different scenarios and show that it has high specificity for a wide range of assumptions regarding the underlying changes in selection, while its sensitivity differs between scenarios. We apply our method to the *env* gene of two HIV-1 subtypes, A and B, and to the HA gene of two influenza A subtypes, H1 and H3, and show that the inferred fitness changes are consistent with the fitness differences observed in deep mutational scanning experiments. We find that changes in relative fitness of different amino acid variants within a site do not always trigger episodes of positive selection and therefore may not result in an overall increase in the frequency of substitutions, but can still be detected from changes in relative frequencies of different substitutions.

Author summary

Which amino acids are acceptable at a certain protein site can change with time. In viruses, for example, this can be due to changes in mechanisms of drug resistance and immune escape in the course of evolution. Here, we develop a method for detecting such changes from how evolutionary events are distributed over an evolutionary tree. Informally, we infer that a certain amino acid is favored in a certain group of lineages if substitutions giving rise to it repeatedly occur in the evolution of this group, and disfavored if such substitutions are rare. In surface proteins of HIV-1 and influenza A, we find that changes in preferences detected with d-test match those observed in deep mutational scanning experiments. Our purely bioinformatic approach allows inference of changes in selection between lineages from sequences alone, shedding light on the functional differences between strains or species even in the absence of any structural or functional data.

Introduction

A particular amino acid at a specific position of a protein may confer different fitness in different species. Such changes in amino acid propensities can occur due to substitutions at other epistatically interacting sites in the course of evolution. Indeed, accounting for co-occurrence of amino acids improves predictions of the rates and patterns of substitutions [1] and polymorphism [2,3], as well as the pathogenic potential of mutations [4,5] and their functional effects in experiments [6–9]. Changes in amino acid propensities can also happen due to shifts in protein fitness landscape caused by a changing environment, e.g. shifts in immune pressure on proteins of pathogens [10–13].

Variability of amino acid propensities has been inferred from statistical analyses of pooled data from numerous amino acid positions of large protein alignments [14,15]. In particular, it has been shown to bias the phylogenetic distribution of homoplasies – repeated substitutions giving rise to the same allele: parallelisms, convergences [16–20] and reversals [21,22]. This is because the fixation probability of a mutation, and therefore the rate of a substitution, giving rise to an allele increases with its conferred fitness [23].

Therefore, substitutions producing the same allele are expected to be more frequent in groups of species where this allele confers high fitness[24]. On a phylogeny, this can be observed as clustering of homoplasies in these groups of species, and such clustering has been interpreted as evidence for changes in fitness landscapes. Existing methods [16–22] aggregate data across multiple sites to increase power, and therefore do not allow to track changes in amino acid propensities at individual sites.

Here, we develop an approach, referred to as the d-test, for identification of amino acid variants at a protein site that confer different fitness in two predefined taxa (such as species or viral strains). This approach uses the phylogenetic distribution of substitutions at this site, and is based on the logic that substitutions that occur in lineages closely related to one of the taxa will be probably more advantageous in it. Using simulations, we show that our method performs well on phylogenies that include hundreds of samples or more. By applying the d-test to large viral phylogenies, we infer sites in the gp160 protein (encoded by the *env* gene) of HIV-1 that have undergone a change in the relative fitness of different amino acids between two strains representative of two highly divergent HIV-1 subtypes; and sites in the hemagglutinin (HA) protein of influenza A that have undergone such a change between two strains representative of two influenza subtypes. We then validate these findings using the fitness differences measured for these strains directly in deep mutational scanning (DMS) experiments [25–27].

Results

Inferring variable fitness amino acids

Using the reconstructed phylogenetic positions of substitutions, we analyze how homoplasic (parallel or convergent, i.e., giving rise to the same amino acid) and divergent (giving rise to different amino acids) substitutions at the same amino acid site are distributed relative to each other over the phylogeny. We propose that an excess of homoplasic substitutions in a set of related lineages implies that the descendant allele confers higher fitness in these lineages, compared to the fitness conferred by it in more

distantly related lineages. By contrast, a deficit of substitutions into an allele implies that it confers lower fitness at this group of lineages [20].

Specifically, we aim to identify individual amino acids at specific sites that arise at significantly different rates between the segments of the phylogenetic tree that contain two predefined nodes ("phylogenetic neighborhoods" of these nodes); these nodes can be external (corresponding to observed species or strains) or internal (corresponding to reconstructed ancestors). For this, we start by considering the first of the two nodes as the "focal" node. For each amino acid at each site, we then test two hypotheses: that the phylogenetic positions of substitutions into this amino acid are biased towards ("proximal amino acids") or away from ("distal amino acids") the focal node (Fig. 1). Next, we consider the second species as the focal node, and repeat the procedure. Finally, we assume that the fitness conferred by an amino acid has changed between the two focal nodes if it was classified as proximal to one of them and as distal from the other, and designate such amino acids as variable fitness amino acids.

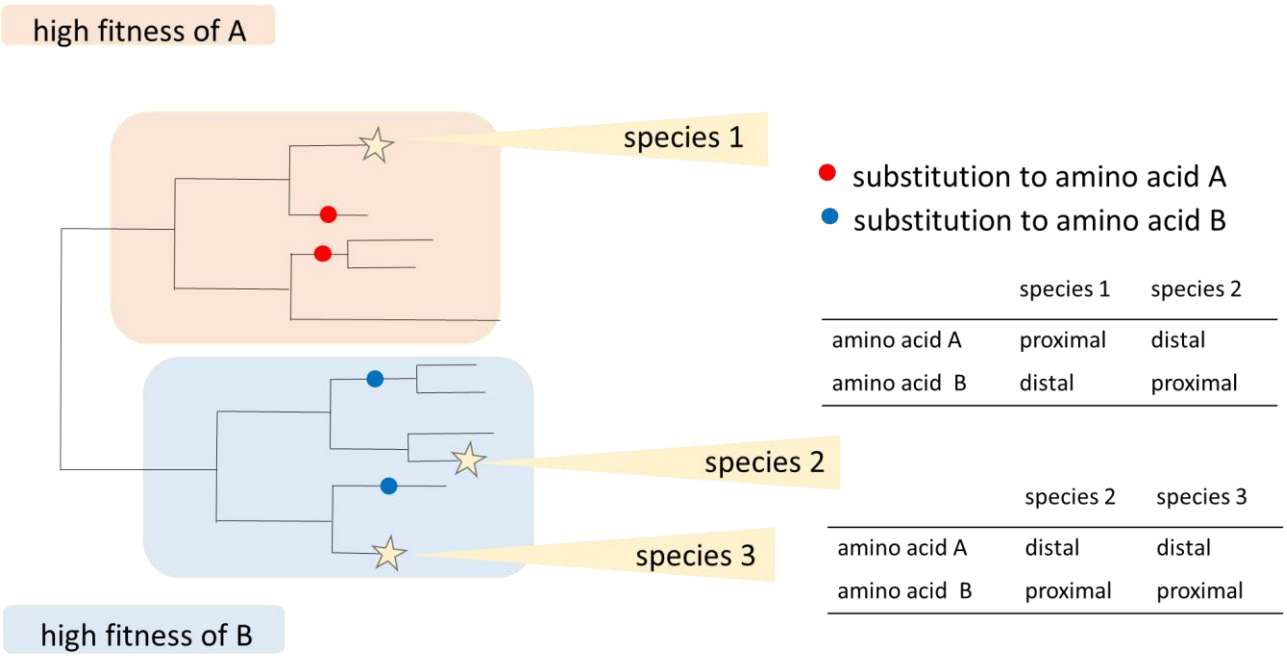


Figure 1. Schematic representation of the approach for inference of changes in fitness of individual amino acids. Both A and B are variable fitness amino acids when species 1 and 2 are chosen as focal nodes, because each of them is proximal to one species and distal from the other. By contrast, A is distal, and B is proximal, for both species in the comparison of species 2 and 3, so neither A nor B is identified as a variable fitness amino acid in this comparison.

Method validation

To understand the power of d-test to identify amino acids with variable fitness, we used SELVa [28] to simulate the evolution of individual amino acid sites under a variable fitness landscape. In our model, the substitution rates between different amino acids were determined by their relative fitness, which could differ between tree branches. Specifically, we assumed that at a given site *a* and at each position of the tree, exactly one of the 20 amino acids, AA_k, is favored by selection and has the scaled fitness $F_a^{AA_k} = 2N_e f_a^{AA_k} = X > 1$, where N_e is the effective population size; while the remaining 19 amino acids have $F_a^{AA_{i\neq k}} = 1$. The $AA_k \longrightarrow AA_{i\neq k}$ substitutions then occur at rate $\frac{1-X}{1-e^{X-1}}$, while the $AA_{i\neq k} \longrightarrow AA_k$ substitutions occur at rate $\frac{X-1}{1-e^{1-X}}$ [29]. To model fitness variability, we assumed that selection changes exactly once at a predefined phylogenetic branch *b;* at this point, the identity of the newly favored amino acid with the fitness X, AA_l, is picked out of the 20 amino acids randomly, and all

remaining amino acids get fitness 1. In the process, the fitness of amino acids AA_k and AA_l has changed, while the fitness of the remaining 18 variants has remained constant.

We asked if the d-test could retrieve the identity of AA_k and AA_l from simulated protein sequences. For simulation, we used the reconstructed phylogenetic tree of the gp160 protein encoded by the *env* gene of HIV-1 (see Methods). The tree contains 3300 tips and has two large clades corresponding to HIV-1 subtypes B and C (Supplementary figure 1). We assumed that selection has changed in the last common ancestor (LCA) of subtype C. To evaluate the dependency of the method performance on sampling density, we additionally ran the simulation for two artificial phylogenetic trees constructed on the basis of the *env* tree: "sparse tree" with roughly half of the tips and "dense tree" with twice as many tips as the original tree (see Methods).

Overall, the simulations indicate that the d-test can detect the variable fitness amino acids with moderate sensitivity and very high specificity (Figure 2 (upper row), Supplementary Table 1). We find that under moderate selection (X = 4 to 9), we correctly identify on average 8%, 43% and 75% of amino acids for which fitness has changed in sparse, original and dense trees, respectively. Under stronger selection (X =10), many sites are monomorphic, and the sensitivity of our method decreases, constituting 24% for the dense tree. Under weaker selection (X = 3), the power of the method also drops, probably because changes in selection of such a low magnitude are masked by the noise associated with drift and tree topology. The percent of amino acids with constant fitness that are identified as positives is low for all trees, never exceeding 0.6%.

As the d-test relies, for each amino acid, on comparing the number of substitutions giving rise to this amino acid and to other amino acids between segments of the phylogenetic tree, it is not applicable if either of these numbers is very low. The set of amino acids to which the d-test can be applied can be identified from the data (see Methods); we refer to them as testable amino acids. When only testable amino acids are considered, the specificity of the test and its sensitivity for weak and moderate selection remain the same,

while its sensitivity for strong selection increases substantially (48% for the dense tree; Figure 2 (bottom row)); this is because we no longer consider the sites where a variant is fixed in the entire clade where it is preferred.

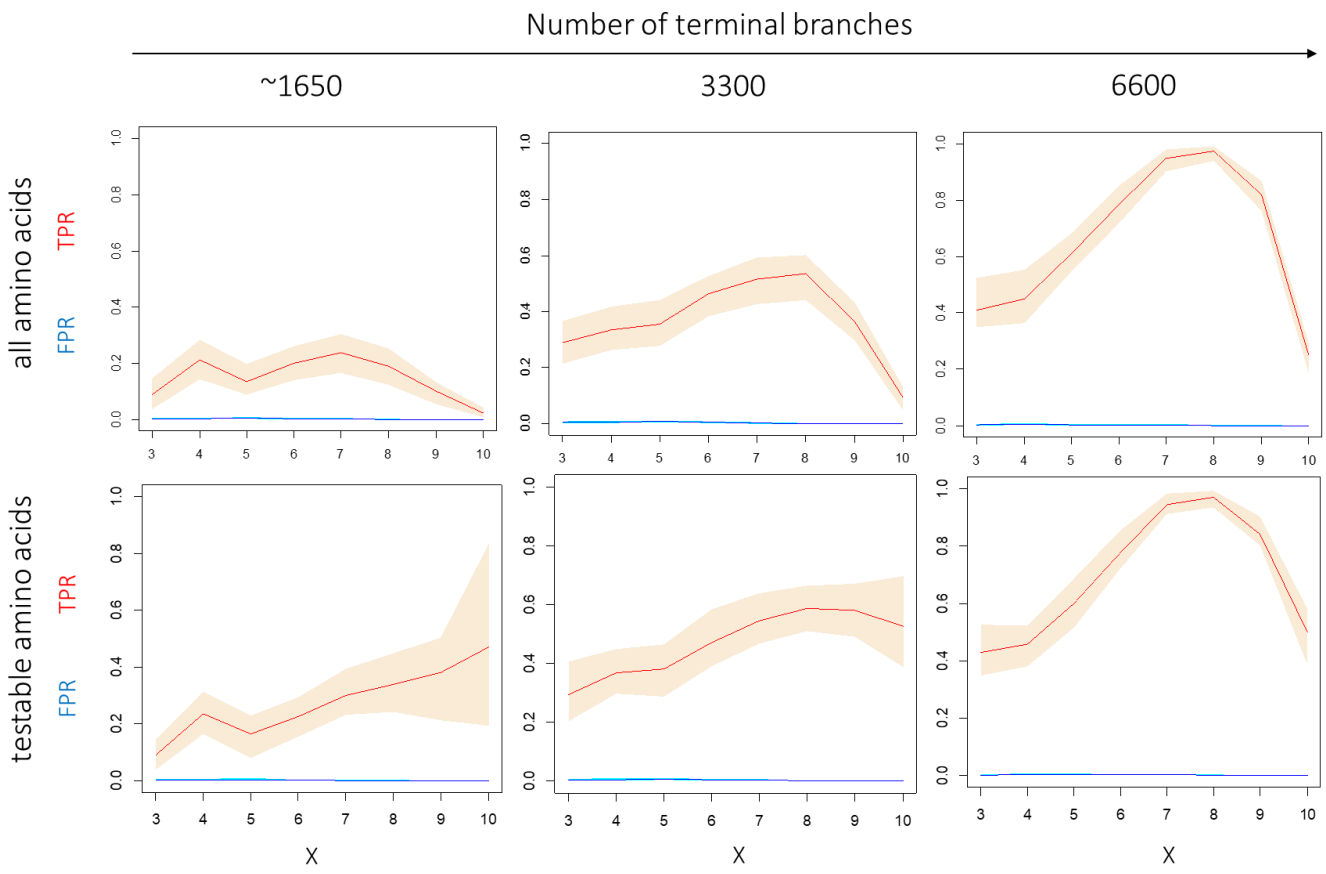


Figure 2. Performance of the d-test for "sparse" (~1650 tips), original (3300 tips) and "dense" (6600 tips) phylogenetic trees in simulations for different scaled fitness values of the preferred variant. Red line, mean True Positive Rate (TPR); blue line, mean False Positive Rate (FPR). 90% confidence bands were obtained by random subsampling of 1000 amino acids in 100 trials. Upper row: all amino acids are considered, bottom row – only testable amino acids are considered. A zoom-in for low FPR values is shown in Supplementary figure 2.

To study the performance of our test under a broader range of conditions, we applied it to simulations under a range of fitness vectors, nodes where selection changes, focal nodes and tree shapes. While the sensitivity of the d-test strongly depends on these parameters, it tends to be high under moderate selection strengths. The specificity of the test was high in all simulations (Supplementary Figure 3; Supplementary Table 2).

Many *env* sites change amino acid preferences in the course of evolution

As a test case, we applied d-test to the evolution of the gp160 protein encoded by the *env* gene in HIV-1. This protein evolves under recurrent positive selection [30,31], suggesting that site-specific amino acid propensities change between strains. As focal nodes, we considered the two strains tested in DMS experiments (see below): BF520 strain of the subtype A and LAI strain of the subtype B.

Among the 3491 amino acids with sufficient variability for our analysis (testable amino acids, see Methods), we detected 59 variable fitness amino acids under the α=0.01 significance cutoff, distributed over 46 of the 724 sites of gp160 (Fig. 3).

As expected, substitutions giving rise to the variable fitness amino acid were positioned on the phylogeny closer to the species for which they were proximal: the phylogenetic distance to that species comprised, on average, 0.87 (range: 0.57 to 0.99, standard deviation = 0.097) of that expected randomly. They also were positioned more remotely from the species for which they were distal: the phylogenetic distance to that species comprised, on average, 1.19 (range: 1.01 to 1.91, standard deviation = 0.19) of that expected randomly.

Of these 59 amino acids, 36 amino acids at 34 sites were proximal to the BF520 strain and distal from the LAI strain; and 23 amino acids at 23 sites were proximal to the LAI strain and distal from the BF520 strain. At 11 sites, changes in fitness were reciprocal: some amino acids were proximal to the BF520 strain and distal from the LAI strain, while others were proximal to the LAI strain and distal from the BF520 strain.

The sites carrying variable fitness amino acids were found at all domains and structural regions of gp120 and gp41, and were not biased towards or away from any functional domain (Fisher's exact test, mean two-sided p-value for functional domains > 0.6).

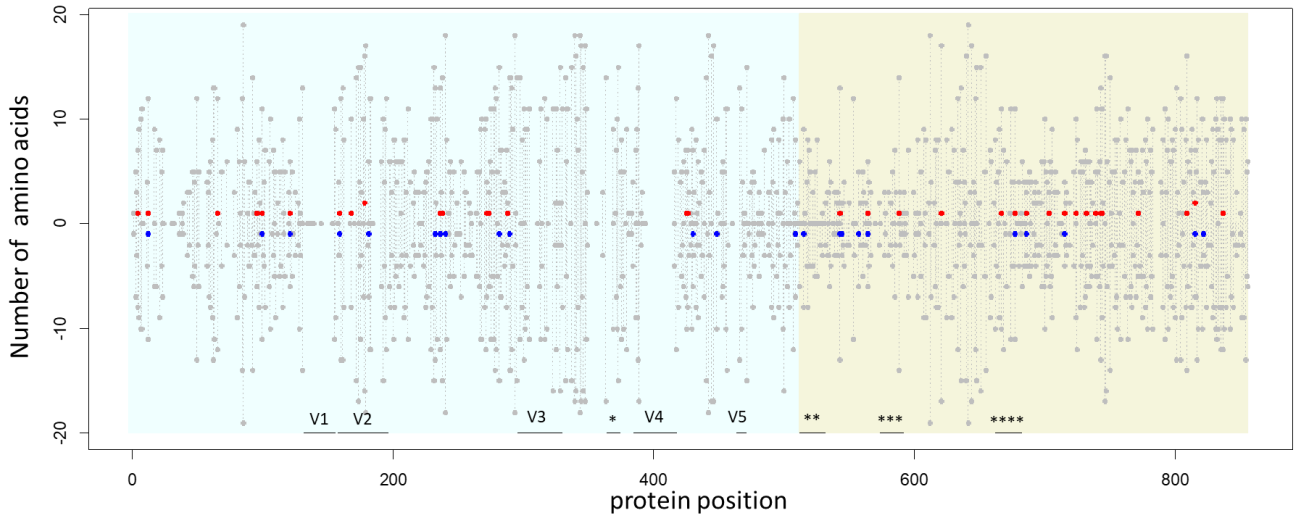


Figure 3. Amino acid sites carrying variable fitness amino acids in gp160 protein of HIV-1. For each position of the amino acid within the protein (horizontal axis), the red dot shows the number of amino acids that are simultaneously proximal to the BF520 strain and distal from the LAI strain; and the blue dot, the number of amino acids that are simultaneously proximal to the LAI strain and distal from the LBF520 strain, multiplied by -1. Grey dots represent the number of testable amino acids (see Methods). The positions without any dots are sites filtered out due to alignment ambiguity. Vertical grey lines connect dots for the same site. Blue background, gp120; green background, gp41. Variable loops V1-V5 and known functional domains are marked by horizontal black lines: *, CD4-binding loop; **, fusion peptide; ***, immunosuppressive region; ****, membrane proximal external region (MPER). Numbering of protein positions is hxb2-based.

Phylogenetically inferred changes in fitness between strains match those observed in DMS experiments

We compared the changes in phylogenetically inferred amino acid preferences between the two strains to those inferred experimentally by DMS [25,26]. To estimate the changes in DMS preferences, for each amino acid, we measured the ratio $r = \pi_x/\pi_y$, where π_x is the DMS preference for this amino acid in the strain for which it is phylogenetically more proximal, and π_y is the preference for it in the strain for which it is more distal. As a control, we analyzed the distribution of r between the two strains for invariable fitness amino acids.

The values of r were significantly higher for the variable fitness amino acids than in the control (one-tailed Wilcoxon's test, p = 0.005; Fig. 4), indicating that the

phylogenetically and experimentally inferred variable fitness amino acids are correlated. This trend is pronounced both for amino acids that conferred higher fitness in BF520 and in LAI strains (Fig. 4). It is mainly driven by amino acids with modest preferences ($\pi < 0.2$; Fig. 4B), which are not the highest-ranking amino acids in DMS experiments in either of the strains (Fig. 4C).

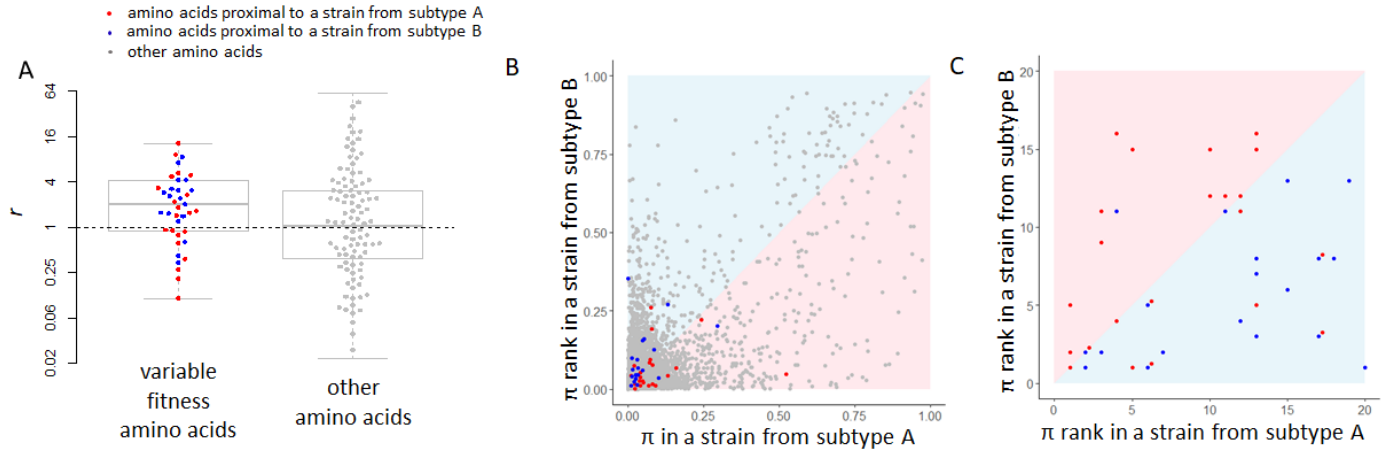


Figure 4. *A*, The ratio of DMS preferences π for an amino acid between the strain for which this amino acid is phylogenetically more proximal and the strain for which it is more distal. Amino acids with significant (left, p<0.01 for both strains) or insignificant (right, p>0.1 for both strains) changes in conferred fitness between strains are shown; bold line is the median, box borders represent the inter quartile range (IQR) between the first (Q1) and the third (Q3) quartiles, whiskers are Q1 - 1.5*IQR and Q3 + 1.5*IQR intervals, outliers are not shown; boxplots are made using all data; each variable amino acid significantly proximal to a strain from subtype A (BF520) or a strain from subtype B (LAI) and a subsample of 100 other amino acids are shown as red, blue and grey dots respectively. The dashed line indicates no change in preferences (r=1). *B*, *C*, Experimentally measured fitness π (B) or fitness rank (C, with rank 1 corresponding to the fittest amino acid) of each amino acid in the two strains of HIV-1. Grey dots, amino acids that have been observed at least in one strain in our phylogenetic tree; red, variable fitness amino acids that are proximal to a strain from subtype A (BF520); blue, variable fitness amino acids that are proximal to a strain from subtype B (LAI); colored areas indicate the expected positions for dots of corresponding color.

Most sites with variable fitness amino acids show no evidence of recurrent positive selection

Changes in fitness conferred by an allele can trigger episodes of positive selection. To study the association between these processes, we identified sites under recurrent positive selection, as evidenced by an above-neutral ratio of nonsynonymous to synonymous substitutions (dN/dS) estimated by codeml ($\omega > 1$) in subtype A, subtype B or both. Among the 46 sites carrying variable fitness amino acids, only 20 (43%) have experienced positive selection in subtypes A or B, despite the fact that the power of both the site-specific dN/dS test for positive selection and our test increases with the number of substitutions. An even lower overlap was observed with the sets of positively selected sites described in previous studies [31:17%,32:9%]. Although there was an excess of positive selection among sites with variable fitness amino acids (Methods; Fisher's exact test, two-sided $p < 0.001$, Table 1), the fact that no such selection was detected in over a half of variable sites suggests that these two phenomena are distinct. Furthermore, r was not higher for variable fitness amino acids at positively selected sites than at sites without any signal of positive selection (one-tailed Wilcoxon's test, $p = 0.09$).

Table 1. Sites with variable fitness amino acids and recurrently positively selected sites.

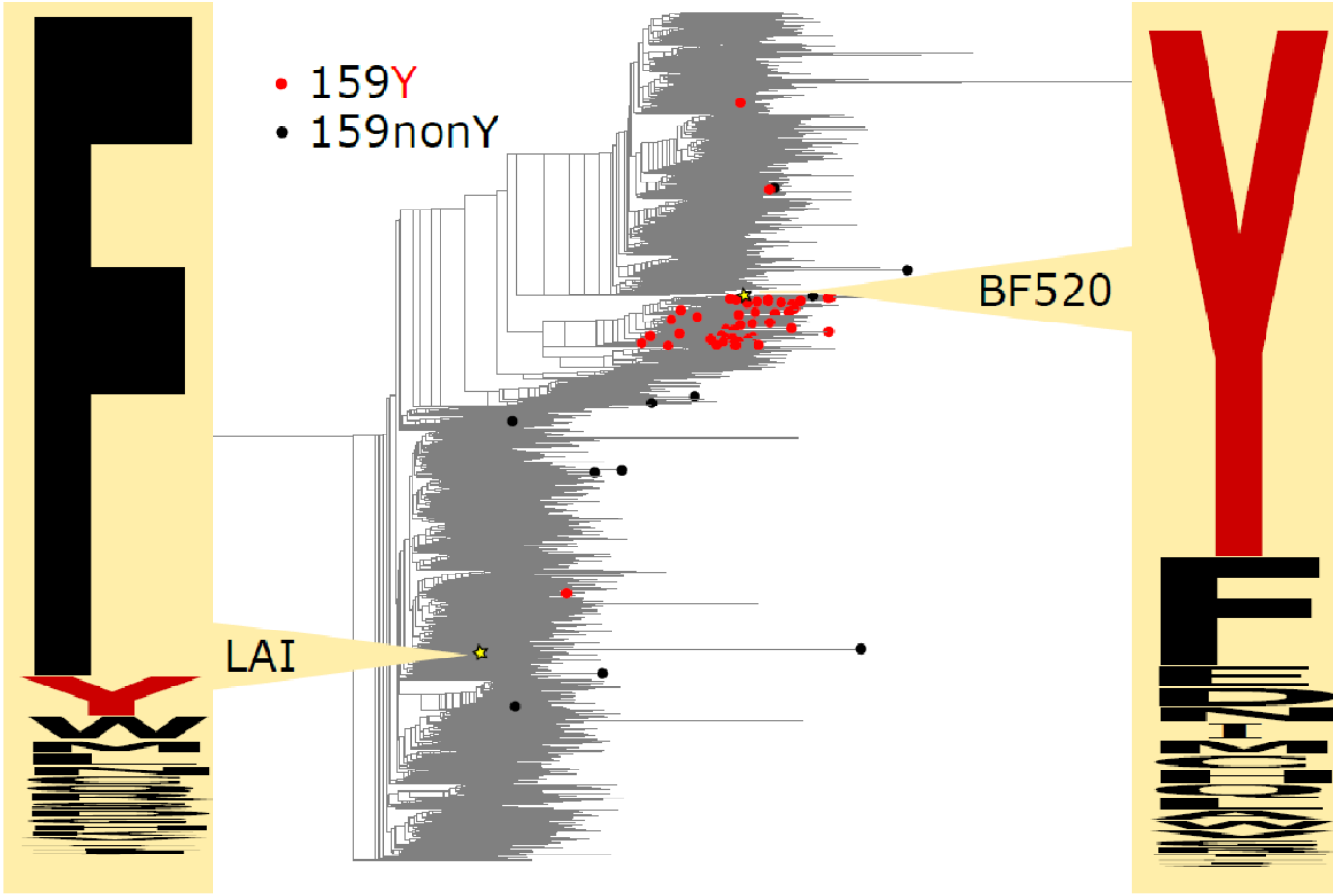
	Sites with variable fitness amino acids	Sites without variable fitness amino acids	p-value*
Our analysis			
Positively selected sites	20	71	<0.001
Other sites	26	422	
[32]			
Positively selected sites	4	21	
Other sites	42	472	0.26
[31]			
Positively selected sites	8	37	
Other sites	38	456	0.04

Only sites with testable amino acids are included. *2-tailed Fisher's exact test

A closer examination of the data helps understand the interplay between the dN/dS ratio and fitness variability. For example, according to the DMS data, Y159 is strongly preferred in a strain from subtype A, while F159 is strongly preferred in a strain from subtype B. In our phylogenetic tree-based analysis, Y159 is a variable fitness amino acid proximal to the strain from subtype A. At site 159, codeml has detected positive selection in subtype A ($\omega = 3.47$), but not in subtype B ($\omega = 0.10$). All substitutions to Y are from F, and within subtype A, F159Y substitutions constitute nearly all (81%) observed substitutions at site 159, so the signal of positive selection picked up by codeml has been created by these substitutions, implying recurrent adaptation (Fig. 5A).

By contrast, while S273 was designated a variable fitness amino acid in our analysis, no positive selection has been detected at site 273 by codeml. All substitutions to S are from R, and R273S substitutions constitute only 5.7% of all substitutions at this site in subtype B, while they account for 46.5% of substitutions in the remaining subtypes (including subtype A). Consistently, S273 confers higher fitness in DMS experiments in subtype A strain than in subtype B strain. Still, even within subtype A, S273 is just one of the several variants with near-equal fitness (Fig. 5B). Therefore, the increased frequency of R273S substitutions in subtype A is likely due to relaxation of negative selection against 273S, rather than positive selection in its favour.

A



B

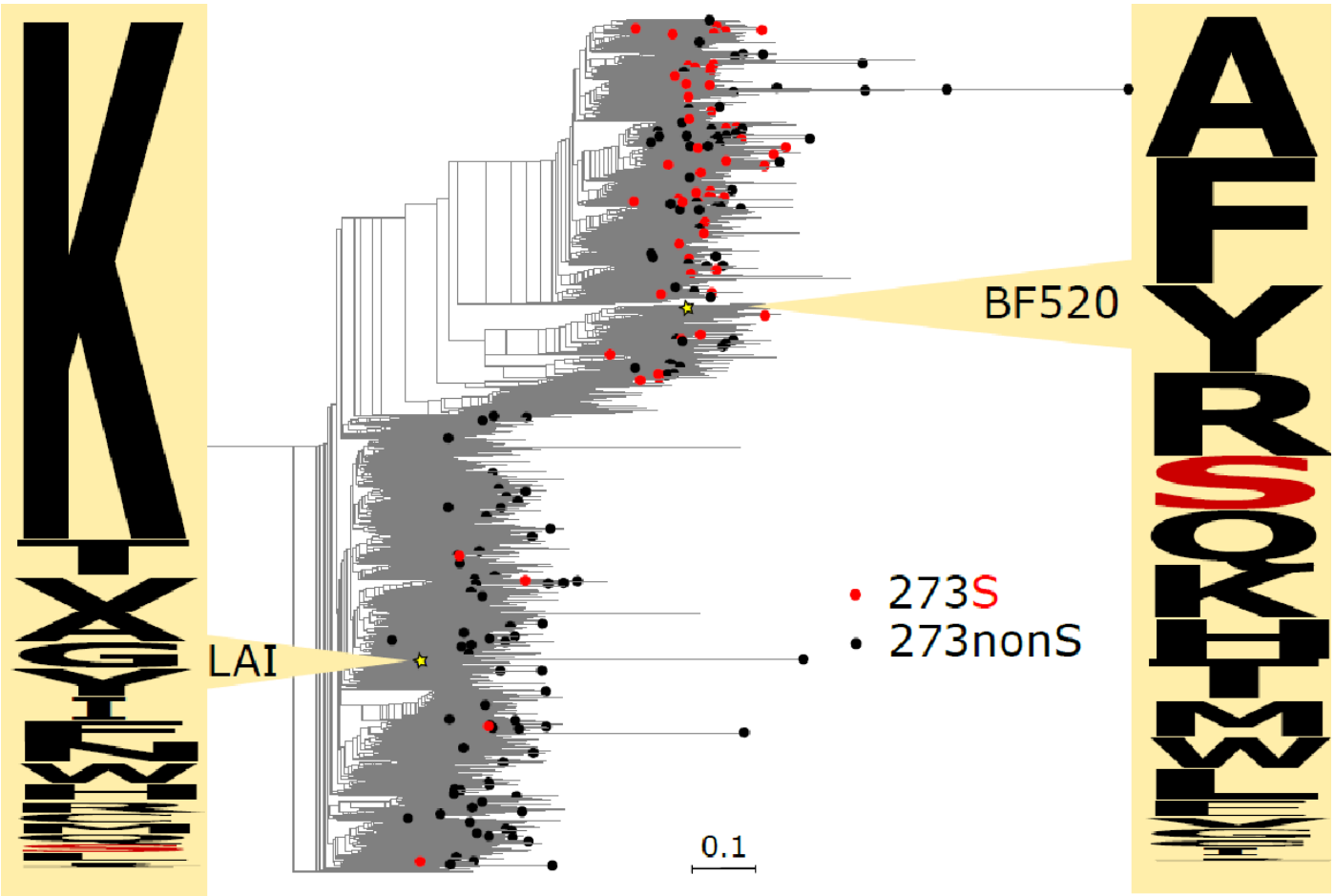


Figure 5. Substitutions to variable fitness amino acids (red) and to other amino acids (black) at sites 159 (A) and 273 (B) of gp-160, and amino acid propensities in DMS experiments in strains BF520 and LAI. Phylogenetic distances are in numbers of amino acid substitutions per site. Numbering of protein positions is hxb2-based. Logos of experimentally derived amino acid propensities were built with Weblogo (https://weblogo.berkeley.edu/logo.cgi).

Differences in fitness constraints between 3 subtypes

Our approach can be extended to detect amino acids with fitness differing in a predetermined pattern between multiple regions of the phylogenetic tree. To illustrate this, we study the amino acids that change the conferred fitness between three strains corresponding to the three major subtypes of HIV-1, A, B and C, which are the most prevalent subtypes in Asia, Northern America/Europe and Africa, respectively. Besides the 59 variable fitness amino acids detected at 46 different sites in the A-B pairwise analysis (see above), we also found 268 additional amino acids that had changed fitness between C and at least one of the subtypes A and B. Overall, we detected 7 amino acids at 5 sites in the A-C comparison, and 285 amino acids at 152 sites in the B-C comparison. These amino acids are listed in Table S1; the web script that lists the variable fitness amino acids between subtypes is available at http://makarich.fbb.msu.ru/galkaklink/hiv_landscape/.

Changes in amino acid propensities may be associated with changes in immunogenicity

To illustrate the potential biological basis of the observed changes in amino acid propensities, we studied how broadly neutralizing antibodies (bnAbs) bind the epitopic sites of gp160. We hypothesized that changes in binding between the major HIV-1 subtypes may be associated with changes in propensities at corresponding sites.

To test this, we considered 9 bnAbs; among those, 6 target the amino acids of gp120 (including VRC01, b12, NIH45-46 which target the CD4-binding site, PG9 which has an epitope in the V1/V2 loop region, and PGT121 and PGT128 that contact the outer domain

of gp120 and the V3 loop base); 1 (2G12) targets glycans in the outer domain of gp120; and 2 (2F5 and 4E10) contact with gp41 (Fig. 6A). For each bnAb, we asked if it differed in inhibition capacity between subtypes. For this, we compared its experimentally measured half maximal inhibitory concentration (IC50) values between subtypes [33,34]. For 5 of the 9 bnAbs, the differences were significant in at least one of the comparisons (Supplementary table 3).

We then mapped the sites carrying variable fitness amino acids in the A-B (46 sites) or the B-C (152 sites) comparison onto the three-dimensional structure of the gp120/gp41 trimer and its complexes with bnAbs. Among these sites, 7 and 28 respectively were epitopic, i.e., were involved in binding with bnAbs according to [35]. (In the A-C comparison, only 5 sites carried variable fitness amino acids, and only one of them was epitopic; these were not analyzed further).

For many of the bnAbs that substantially differed in binding between two subtypes, we found that the associated epitope carried some sites with variable fitness amino acids between these subtypes (Supplementary table 3). For example, the two epitopes that are responsible for differential binding between subtypes A and B (those in the V1/V2 region and the CD4-binding site) both carry amino acids with variable fitness between these two subtypes, while the remaining of the gp120 epitopes (the one in the V3 loop) carries no such amino acids and experiences the same level of binding between the two subtypes (Fig. 6B).

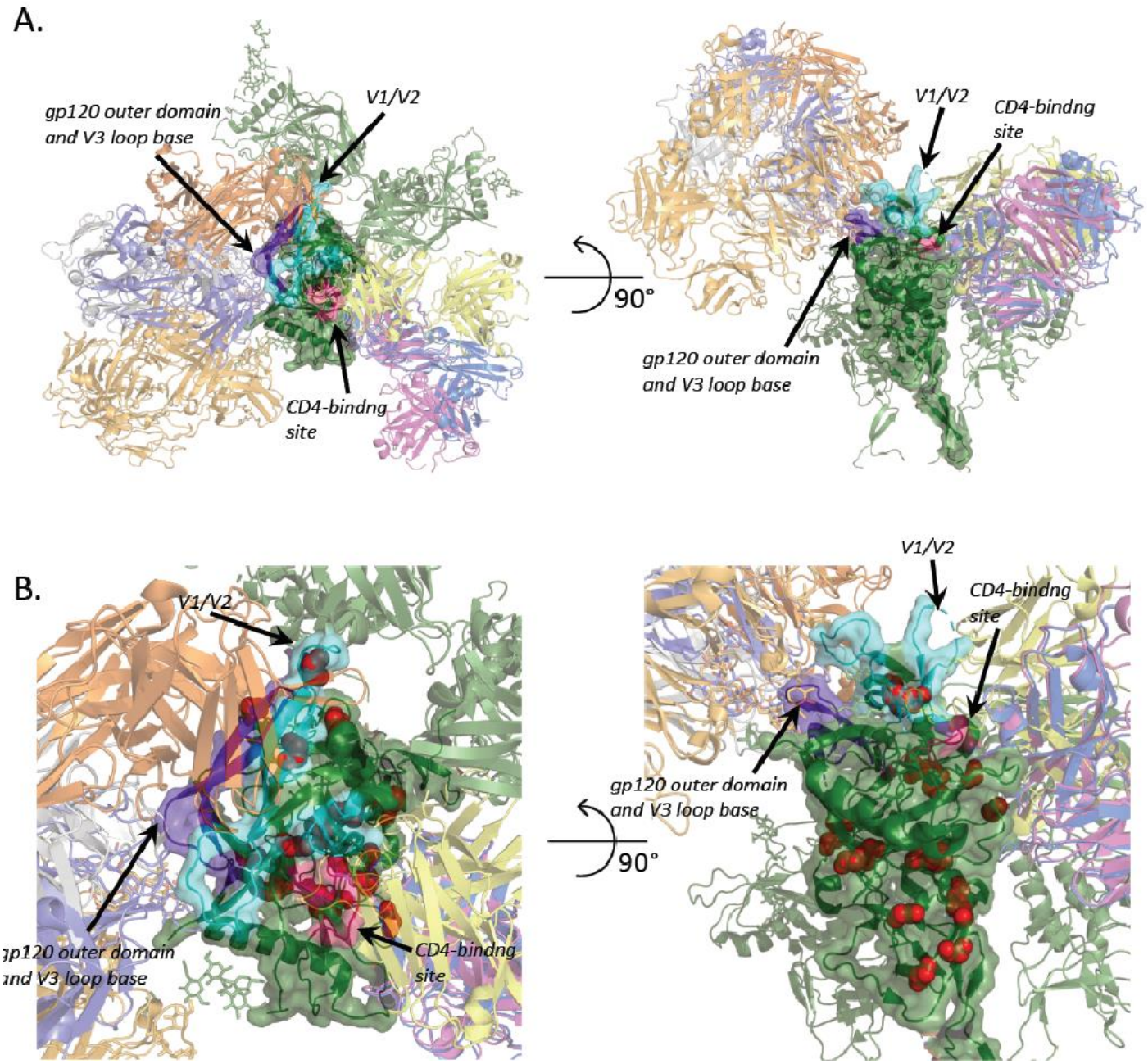


Figure 6. (A) Overview of the structure of the gp120 trimer and its complexes with bnAbs (see Methods). gp120 is colored green and shown as cartoons, one subunit is also shown as a semi-translucent surface. bnAbs are in various color and shown as cartoons. Glycan molecules are shown as sticks. bnAbs binding only one gp120 subunit are shown for clarity. (B) Close-up of one gp120 subunit, with epitopes signed; sites with variable fitness amino acids between subtypes A and B shown as red spheres. Epitopic sites are defined according to [35]; CD4-binding site is colored pink, V1/V2 loop region cyan, and V3 loop region purple.

This suggests that shifts in fitness landscapes of epitopic sites may be associated with changes of antigenic properties of these epitopes. Remarkably, in 16 out of 18 sites that carry variable fitness amino acids and reside in epitopes differentially bound by antibodies, the identity of the amino acid that actually occupies the subtype consensus remains the same between the two subtypes, despite changes in propensities

(Supplementary table 4). This suggests that the differences in binding may be associated with changes in fitness conferred by the incumbent and/or other amino acid variants without an actual amino acid replacement. This suggests that the differences in binding may be associated with changes in relative fitness of amino acids in epitopic sites that did not lead to change in identity of the most prevalent amino acid.

Variable fitness amino acids in HA of Influenza A

We applied the d-test to the HA gene of H1 and H3 subtypes of human influenza A and detected 3 variable fitness amino acids under the α=0.01 significance cutoff. Although statistically our results did not significantly correlate with the experimental fitness measures (one-tailed Wilcoxon test, p = 0.08), all three amino acids had higher experimental fitness in strains for which they were proximal than in those for which they were distal. Under a higher significance cutoff (α=0.05), we detected 9 variable fitness amino acids at 7 sites (Fig. 7).

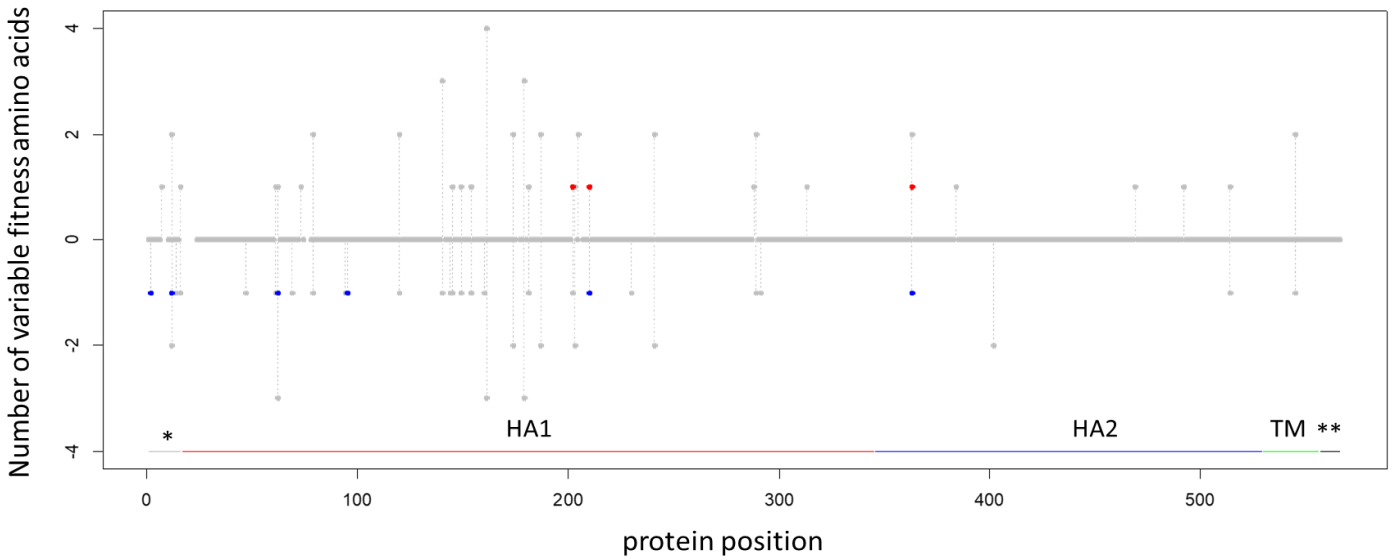


Figure 7. Amino acid sites carrying variable fitness amino acids in HA of influenza A virus. For each protein position (horizontal axis), the red dot shows the number of amino acids that are simultaneously proximal to the H1 strain and distal from the H3 strain, and the blue dot, the number of amino acids that are simultaneously proximal to the H3 strain and distal from the H1 strain, multiplied by -1. Grey dots represent the number of testable amino acids (see Methods). The positions without any dots are sites filtered out due to alignment ambiguity. Vertical grey lines connect dots for the same site. Protein domains are marked by horizontal lines: *, signal peptide; HA1, HA1 ectodomain; HA2, HA2

ectodomain; TM, transmembrane domain; **, cytoplasmic tail; numeration of protein positions is A/Perth/16/2009 based.

The identities of these 9 amino acids were again consistent with the DMS experiment: amino acids with an excess of substitutions into them in the phylogenetic neighborhood of one of the strains conferred higher fitness at this strain than in the other strain (one-tailed Wilcoxon's test, p = 0.02; Fig. 8).

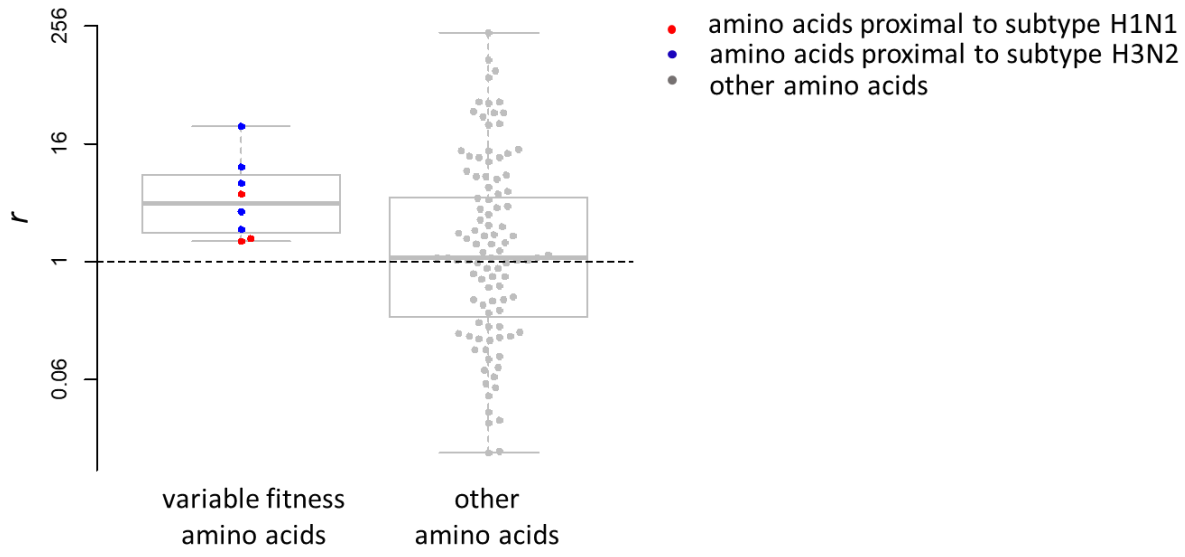


Figure 8. The ratio between experimentally measured amino acid preferences of the strain for which an amino acid is more proximal and the strain for which it is more distal in HA of influenza A. Amino acids with significant (left, p<0.05 for both strains) or insignificant (right, p>0.1 for both strains) changes in the phylogenetic analysis are shown; notations are the same as in Figure 4.

For some of the sites, changes in fitness could be interpreted from the functional perspective. For example, site 88 (A/Puerto Rico/8/1934 numbering) is antigenic in H1, but not in H3 [36]. We suggest that variability in fitness of amino acid L that we found reflects differences in the amino acid propensities at this site between the subtype where it is epitopic (H1; Fig. 9A) and subtype where it is not epitopic (H3; Fig. 9B). Our suggestion is supported by the fact that 7 amino acids can be observed at this site in H1, but only 2 in H3.

Another example is site 202 (in A/Perth/16/2009 numbering) that was previously shown to be antigenic in H3, but not in the H1 subtype [37]. At this site, S is the ancestral amino acid

for both H1 and H3 clades. Both our analysis and the DMS experiment suggest that P202 confers higher relative fitness in H1 than in H3. Four S202P substitutions have occurred in the H1 clade, and 90% of the H1 isolates contain P202. Conversely, no substitutions into P have occurred in the H3 clade, and 95% of H3 isolates carry G202, which has originated from S in a total of 3 substitutions. The observed differences in substitution patterns might reflect the differences in the function of site 202 between the two viral subtypes (Fig. 9).

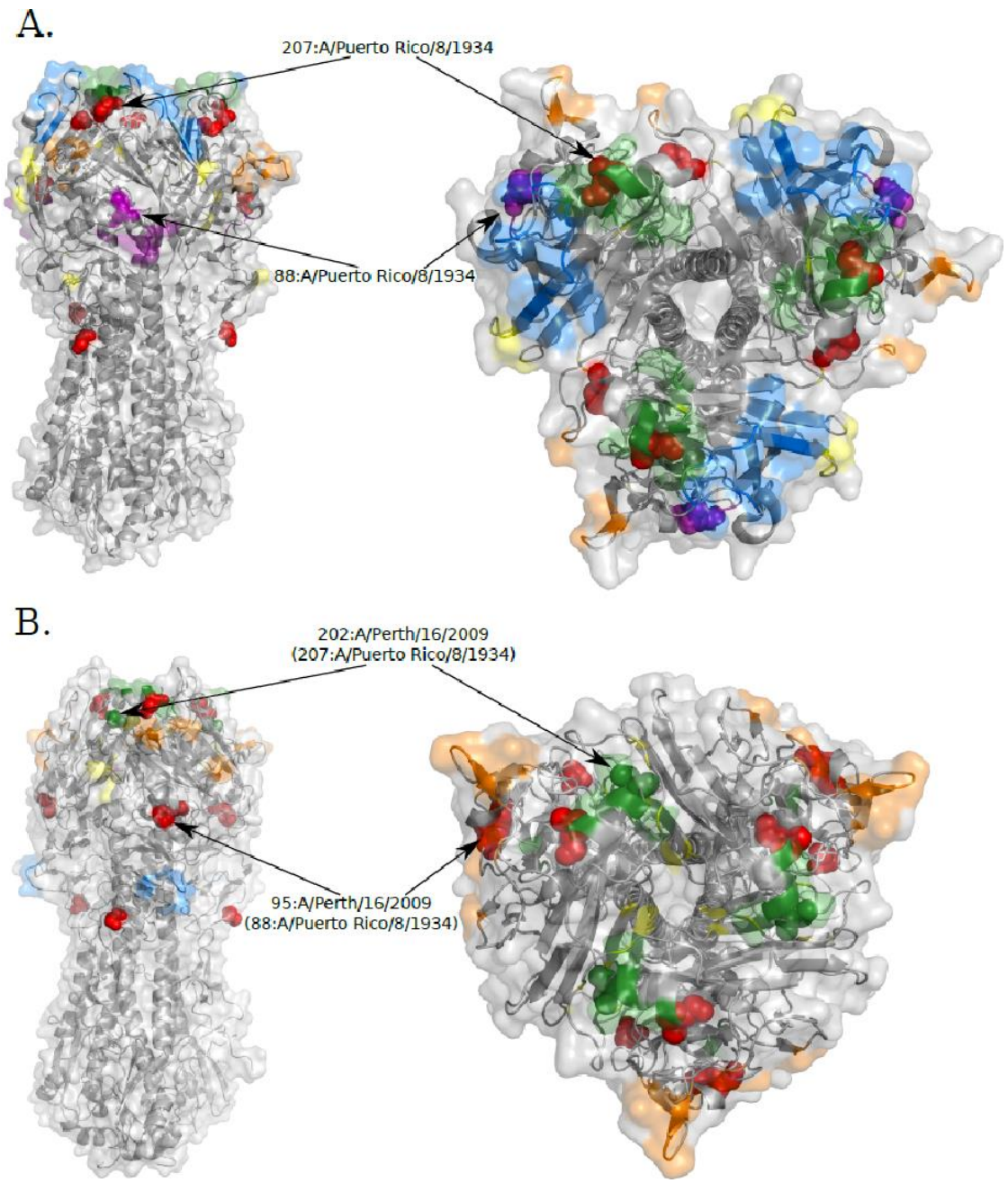


Figure 9. Variable fitness sites in the structure of the Influenza hemagglutinin. Sites with variable fitness amino acids are shown as red spheres except when they are located in one of the antigenic sites. (A) Subtype H1 trimer (PDB id 1ru7). Antigenic sites were mapped based on [36]: Ca1 is shown in yellow, Ca2 in orange, Cb in magenta, Sa in blue,

Sb in green. (B) Subtype H3 trimer (PDB id 2viu). Antigenic sites were mapped based on [37]: A is shown in orange, B in green, C in blue, D in yellow.

Discussion

Patterns of substitutions in the course of evolution of a protein are shaped by the selection pressure experienced by it, and changes in this pressure may lead to non-uniformity of substitution rates between evolving lineages. While the overall non-stationarity of evolutionary rate [heterotachy, 38–40] as well as of individual substitution types [heteropecilly, 19,20,41] have been used to infer changes in underlying selection, these approaches require pooling multiple sites to obtain a statistical signal. Therefore, they are unable to pick up changes in amino acid propensities within individual sites, especially if the directions of these changes were discordant between sites.

Large sequence datasets with inferable phylogenetic relationships can be harvested to increase the resolution of these methods. Here, we develop an approach to infer, for individual amino acids at individual sites, the changes in relative fitness that they confer between two related lineages.

Our method (the “d-test”) makes use of changes in relative frequencies of convergent (or parallel) and divergent substitutions between evolving lineages. While the high frequency of substitutions giving rise to the same amino acid suggests a high fitness of that amino acid, usage of divergent substitutions provides an internal control, making the d-test robust and insensitive to other types of non-stationarity. In particular, first, it is not sensitive to differences in the overall substitution rate (across sites and mutation types) between lineages. Second, as it considers each site individually, it is not sensitive to differences in substitution rates or patterns between sites. Third, as it controls for the identity of the ancestral amino acids, it is not sensitive to differences in rates of different types of substitutions (e.g., transitions vs. transversions) within a site that are conserved throughout the considered phylogeny. Fourth, for the same reason, it is not sensitive to

changes in the rate of the substitutions of the same type that are caused by the changes in the prevalence of a particular ancestral amino acid [20].

Still, our method has some limitations, most of which have been discussed previously [20]. First, as it is based on the analysis of phylogenetic distribution of amino acid substitutions, its power is limited by the number of substitutions observed, which in turn depends on the tree shape and the selection regime. In particular, it is inadequate for detection of changes in amino acid fitness between closely related species or at slowly evolving sites. Here, we explicitly study the power of the d-test for different selection strengths. Simulations show that our method works best within a window of selection coefficients corresponding to moderate selection. This is to be expected. If selection is too weak, both weakly advantageous and weakly deleterious substitutions occur throughout the phylogeny, making our approach impotent. Conversely, if selection is very strong, the number of variable sites becomes low: the favored amino acid is usually nearly fixed in one of the clades, so that there are no substitutions giving rise to it to analyze. In particular, the d-test is inadequate for detection of selection in favour of a highly conserved ancestral variant, since multiple substitutions into this variant are unlikely. Furthermore, the power of the d-test increases when sequences of more species are available (Fig. 2). This happens for two reasons: first, an increase in the number of substitutions gives more power; second, even with the same number of substitutions at a site, the power is higher for sites with more distinct ancestral amino acids, as at such sites, there are more combinations of substitutions in the null distribution, and thus a lower minimal p-value can be obtained.

Second, the d-test is based on the assumption that the similarity between the substitution patterns (which are determined by amino acid propensities) is associated with the distance between the lineages in the phylogenetic tree; in other words, more closely related lineages are more likely to share the amino acid propensities. While this assumption probably holds on average, it can be easily violated in specific cases: for example, propensities can be shared with more remote lineages while they can be different from those in closer ones, depending on the phylogenetic position of the considered species

and details of the tree shape (Supplementary Figure 4). When this assumption is violated, the performance of the test will drop (Supplementary Table 2). This problem can be alleviated by only including in the analysis the minimal clade containing the two focal species, and excluding more remote species that can confound the signal (Supplementary Table 2).

Third, our test detects any change in the frequency of an individual substitution at a site independent of its cause. While at the amino acid level, such changes are probably always caused by changes in amino acid propensities, if a similar approach is applied to nucleotide substitutions, rate changes can also occur due to differences in mutation biases or in non-amino-acid level selection, e.g. selection for nucleotide composition. In theory, synonymous sites can be used to control for non-selective changes in substitution biases, although this is not straightforward for viruses where many of such sites can be constrained by selection on structure or overlapping reading frames. However, mutation spectra are similar between HIV-1 subtypes [42], strongly suggesting that the changes we detect are caused by differences in selection rather than mutation.

As the probability of a substitution to an amino acid depends on its N_e-scaled relative fitness, theoretically, the d-test could be sensitive to differences in N_e between subtypes. We are unaware of any data showing systematic differences in N_e between HIV or influenza A subtypes. More generally, changes in N_e should affect all sites concordantly, and it is difficult to imagine a scenario whereby they will lead to changes in density of homoplasies of opposite directions between sites, as observed in our analysis.

Fourth, our method relies on the correctness of the inferred phylogeny. As significant, we only consider those sites that are reproduced in 10 out of 10 independently reconstructed phylogenetic trees, and therefore believe that our findings are robust to errors in overall shape of the gene tree. Incongruence between phylogenies of the gene and the considered site can also result from within-gene recombination. While we exclude known recombinants from analysis, some recombination events, particularly within-subtype ones, could have been undetected. To result in spurious clustering of

homoplasies, such recombination would have to be recurrent (as each recombination event can lead to at most one spurious homoplasy), and to preferentially affect closely related lineages (as no clustering would be observed otherwise). However, it is unclear why random recombination would specifically favor amino acid variants that have high experimentally measured fitness in the corresponding clade.

To illustrate and experimentally validate our approach, we make use of two DMS datasets for viral surface proteins, in each of which the amino acid preferences have been measured experimentally for two different strains: the gp160 protein in two HIV-1 strains, and the HA protein in two influenza A strains. Overall, we find that the results of the phylogenetic test are consistent with experimental results. If a particular amino acid is gained more frequently by a species closely related to a particular strain, it also tends to confer higher fitness in experimental assays in that same strain.

We find that the inferred variable fitness amino acids mostly have modest experimentally measured preference π even in the strain for which they are proximal. There can be multiple reasons for this. First, our phylogenetic test is not guaranteed to assign high preference to the fittest amino acid at a site. In particular, it is only able to assign differential fitness to derived, but not ancestral, variants. For example, 159F confers high experimentally measured fitness in the gp160 of the LAI strain, but its fitness is lower in the BF520 strain (Fig. 5A); still, it cannot be picked up by the phylogenetic test, as it is the ancestral variant, and the substitutions giving rise to it are rare in both strains (Fig. 5A). As the variant that is much more fit than all other variants is likely to be conserved throughout the clade, the phylogenetic method is less likely to pick it up, compared to a variant with fitness comparable to that of at least one other variant.

Second, many of the fitness shifts between A and B subtypes of HIV-1 did not affect the fittest amino acid. Indeed, among the 42 amino acids with significant phylogenetically inferred fitness shifts between these subtypes, only 8 (19%) had the highest DMS-inferred fitness rank in either strain (Fig. 4C). The remaining 34 amino acids were suboptimal in both strains. Therefore, the d-test allows to infer the changes in fitness between

suboptimal amino acids, which is relevant for understanding the overall fitness landscape governing the evolution of the protein.

An evolutionary model that incorporates the results of a DMS scan [ExpCM; 43] has been shown to outperform other substitution models, and, in particular, detect positive selection more robustly. This is achieved by obtaining the strength of functional constraint, indicative of negative selection, for individual sites from the DMS data [26,27]. In line with that model, the changes in substitution rates observed here can be caused by changes in the strength of negative selection between strains. Additionally, changes in amino acid preferences can trigger episodes of positive selection if the newly preferred amino acid is rare in the ancestral population. We find that less than a half of sites with variable fitness amino acids also had signatures of positive selection that could be detected by a standard dN/dS approach. Therefore, changes in site-specific fitness landscape are not necessarily coupled with strong positive selection favoring new optimal variants.

Our method can be applied to more than two focal nodes to find amino acids that have the same relative fitness in some lineages but change it in other lineages. We illustrate this using the three subtypes of HIV-1. As the number of strains substantially differs between these three clades (223, 2035 and 1265 for A, B and C correspondingly), the power of the d-test differs between them. Still, our results show that with enough data we are able to compare fitness of an amino acid between more than two clades. Importantly, unlike other methods for detection of changes in selection preferences, our test can be applied without prior identification of candidate clades differing in selection pressure [44], and makes no assumption regarding model of epistatic interactions or fitness shift [44,45].

Predicting the effects of genetic variation on fitness is often based on the extent of conservation of a position [46–48], but functionally important residues are not necessarily conserved [49]. Our method can be used to distinguish between rapidly evolving sites with relaxed and variable constraints. For example, in gp160, there are 10 sites each with

18 different amino acids that originated at some branch(es) of the phylogenetic tree. While this is suggestive of complete neutrality of these sites, we find that, among these 10 sites, 5 carry variable fitness amino acids.

Despite the recent surge in DMS experiments that allow high-throughput fitness measurements, such experiments can still only be performed for a relatively few model systems, and are still impractical for large numbers of strains. Moreover, the fitness measurements in experimental conditions are not necessarily reflective of the selective pressure experienced by a lineage over the course of its evolution [25]. By contrast, comparative genomics allows to assess the fitness differences that have shaped evolution for a large number of non-model species simultaneously.

Materials and Methods

Inference of phylogenetic biases (principle of the d-test)

We define the phylogenetic distance d between the focal nodes and each substitution into amino acid A as the sum of branch lengths along the path between them on the phylogeny, assuming that a substitution occurs at the middle of the corresponding branch, and $\bar{d}$ as the mean of those distances. If the substitutions preferentially occur in the phylogenetic neighborhood of the focal nodes, $\bar{d}$ will be lower than expected. By contrast, if substitutions are underrepresented in the phylogenetic neighborhood of the focal nodes, $\bar{d}$ will be higher than expected. To obtain the null distribution of $\bar{d}$, we make use of the substitutions into non-A amino acids. $\bar{d}$ depends both on the phylogenetic distribution of the amino acids ancestral to A and on the number of substitutions into A [20]. To control for this, we subsampled the substitutions from the same ancestral amino acids as follows. We listed all "ancestral" amino acids that were substituted by A at least once. For each such ancestral amino acid, we then considered all substitutions into any (A or non-A) amino acid, and subsampled, out of these substitutions, the number of substitutions equal to that of substitutions into A. We then pooled these substitutions across all ancestral

amino acids together, and measured the mean phylogenetic distance $\tilde{d}$ for them. This subsampling was repeated 10,000 times to obtain the null distribution of $\bar{d}$.

The p-value for the bias of substitutions towards the focal nodes was defined as the percentile of $\bar{d}$ in this distribution, and for their bias away from the focal nodes, as 1 minus this value. We required $p < \alpha$ for proximal amino acids, and $p > (1-\alpha)$ for distal amino acids, where α is the significance threshold. An amino acid was considered "testable" (proximal or distal) if the minimal attainable p-value (i.e., the fraction of the values of $\bar{d}$ in the most extreme bin) was lower than α. For each significance threshold α, we calculated the expected fraction of false positives (Q-value) as min $(\alpha*P)/V$, where P is the number of testable amino acids at this α, and V is the number of significant amino acids at this α. We used $\alpha=0.01$ for gp160 throughout; the percent of expected false positives at this p-value is less than 12% (Supplementary Figure 5). For HA, where the substitution rate is lower, which led to more than 50 times lower P at $\alpha = 0.01$, we used $\alpha=0.05$, yielding the Q-value<62% (Supplementary Figure 5).

Finally, for a pair of species 1 and 2, we defined an amino acid as "variable fitness amino acid" if it was proximal for one species and distal for the other one. All amino acids that were potentially significantly proximal for one species and potentially significantly distal for the other were considered as "testable amino acids".

All scripts needed to run the d-test along with Manual can be found on GitHub: https://github.com/GalkaKlink/d-test.

Data

gp-160

We downloaded 4241 gp160 DNA and protein sequences belonging to nine subtypes of HIV-1 from the Los Alamos HIV sequence database (http://www.hiv.lanl.gov/) using the following settings: alignment type=filtered web, organism=HIV-1/SIVcpz, region=Env, subtype=M group without recombinants, year: 2016. The subtypes were represented unequally, with the majority of sequences from subtypes B (>2000 isolates) and C (>1000

isolates). We discarded sequences with lengths not divisible by 3 and internal stop codons, leaving 3789 sequences. We then added two experimentally studied sequences LAI and BF520, and an outgroup type O (isolate_ANT70), for a total of 3792 sequences. Using Pal2Nal [50] and Mafft [51], we obtained amino acid alignments and codon-informed nucleotide sequence alignments. Using nucleotide alignments, we built a maximum likelihood phylogenetic tree using RAxML under the GTRGAMMA model. We then optimized branch lengths on the basis of the amino acid alignment under the PROTGAMMAGTR model [52] and reconstructed ancestral states with codeml program of the PAML package [53].

The analysis was performed on amino acid alignments, using LAI or BF520 as focal nodes. To ensure that our results are robust to uncertainty of phylogenetic reconstruction, for all analyses involving one "proximal-distal" comparison, we constructed maximum likelihood phylogenetic trees on the basis of the same alignment in 10 independent runs, identified the variable fitness amino acids for each tree, and performed downstream analyses with just those amino acids that had significant shifts in fitness in all 10 replicates.

Functional regions were defined as annotated in UniProt [54; entry P04578]. We obtained solvent accessibility data for the LAI strain from [25]. To define buried and exposed residues, we used the threshold relative solvent accessibility = 0.25.

Hemagglutinin

We obtained 2238 and 50,109 DNA sequences of HA from seasonal H1N1 and H3N2 influenza A subtypes correspondingly from the GISAID database [55]. We discarded sequences shorter than 1680 nucleotides, with lengths not divisible by 3 or with internal stop codons, leaving 2142 sequences of H1 and 49543 sequences of H3. We then used cd-hit program [56] to discard identical sequences from the dataset, leaving 1557 sequences of H1 an 20191 sequences of H3. Finally, we randomly subsampled 1557 sequences of H3 to make the H1 and H3 datasets equal in size. Protein sequences were obtained from DNA sequences with BioPerl scripts. All subsequent procedures were

conducted as for the HIV-1 dataset. We obtained experimentally derived amino acid propensities for A/WSN/1933 (H1N1) and A/Perth/16/2009 (H3N2) strains from [27].

Simulated evolution

We simulated evolution of the protein across the phylogeny of two HIV-1 subtypes, B and C. The phylogeny was constructed as above, but only sequences from B and C subtypes were used. The "sparse" tree was constructed by removing a fraction of terminal branches from the real tree, and the "dense" tree, by adding, to each terminal node of the initial tree, two new branches ending with new terminal nodes, with lengths uniformly picked from the range (0;0.2]. We made these trees using BioPerl functions (https://bioperl.org/).

Simulations were performed with the Simulator of Evolution with Landscape Variation (SELVa) program [28]. SELVa allows to specify the branches of the phylogenetic tree where a fitness shift occurs, and the vectors of amino acid fitness before and after the shift. It simulates substitutions between amino acids according to the model described in [29]. The mutation rates are assumed to be uniform, so the differences in substitution rates are solely determined by differences in fitness.

We simulated evolution of 500 sites along the phylogenetic tree of HIV-1 with the amino acid fitness vector $\{X,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1\}$, for each integer X between 3 and 10 . The identity of the amino acid with fitness X differed between subtypes B and C; the corresponding fitness shift was assumed to have occurred in the last common ancestor of subtype C.

For each X, we calculated two values: the true positive rate (TPR) – the proportion of amino acids, among those that have changed fitness in the course of evolution, that were correctly identified as positives; and the false positive rate (FPR) – the proportion of amino acids, among those that have not changed fitness, that were incorrectly identified as positives. These values were calculated for 1000 randomly sampled amino acids for 100 times to obtain the mean and the 90% confidence interval.

Comparison with DMS data

To test whether our results match the results of the DMS experiments, for each amino acid, we measured the ratio r between the DMS preference for this amino acid in the strain for which it is phylogenetically more proximal (π_x) and in the strain for which it is more distal (π_y). The proximity to one or the other strain was determined by comparison of the corresponding p-values, independent of their significance, so that all amino acids could be thus classified. We then compared the mean r for variable fitness and other amino acids with Wilcoxon's rank sum test.

Identifying sites under positive selection

Sites of gp160 were tested for positive selection using the codeml program of the PAML package with site model. The presence of positive selection was tested by comparison of models M1a and M2a with the likelihood ratio test. Probabilities that positive selection acts at each site were calculated by BEB (Bayes Empirical Bayes) method as implemented in codeml. We separately performed the analysis for subtype A subtree, subtype B subtree and A+B tree. The trees for these analyses were built as described above, but only sequences from subtypes A and/or B were used.

Studying association of variable fitness amino acids with differences of antigenic properties between HIV1 subtypes

We used IC50 values for 19 subtype A strains, 31 subtype B strains, and 28 subtype C strains for bnAbs VRC01, NIH45-46, b12, PG9, 2G12, 2F5, and 4E10 from [34]; for 8 subtype A strains, 13 subtype B strains, and 12 subtype C strains for bnAbs PGT121 and PGT128 also from [34]; and for 31 subtype A strains, 48 subtype B strains, and 59 subtype C strains for bnAbs VRC01 and PG9 from [33]. For each bnAb, we performed a two-sided Mann-Whitney test to understand whether its neutralising capacity differs between subtypes A and B or subtypes B and C.

Then we mapped sites with the variable fitness amino acids onto the overlay of multiple gp120/bnAb complexes: with VRC01 (PDB id 3ngb), PG9 (PDB id 3u4e), b12 (PDB id

2ny7), NIH45-46 (PDB id 4jkp), 2G12 (PDB id 6e5p), PGT121 (PDB id 5c7k), and PGT128 (PDB id 5cez). Epitopic sites were defined according to [35].

To show all considered antibodies in connection with gp120 on one picture (Fig. 6A), we combined structures 6e5p, 4jkp, 3ngb, 2nz7, 5cez and 5c7k.

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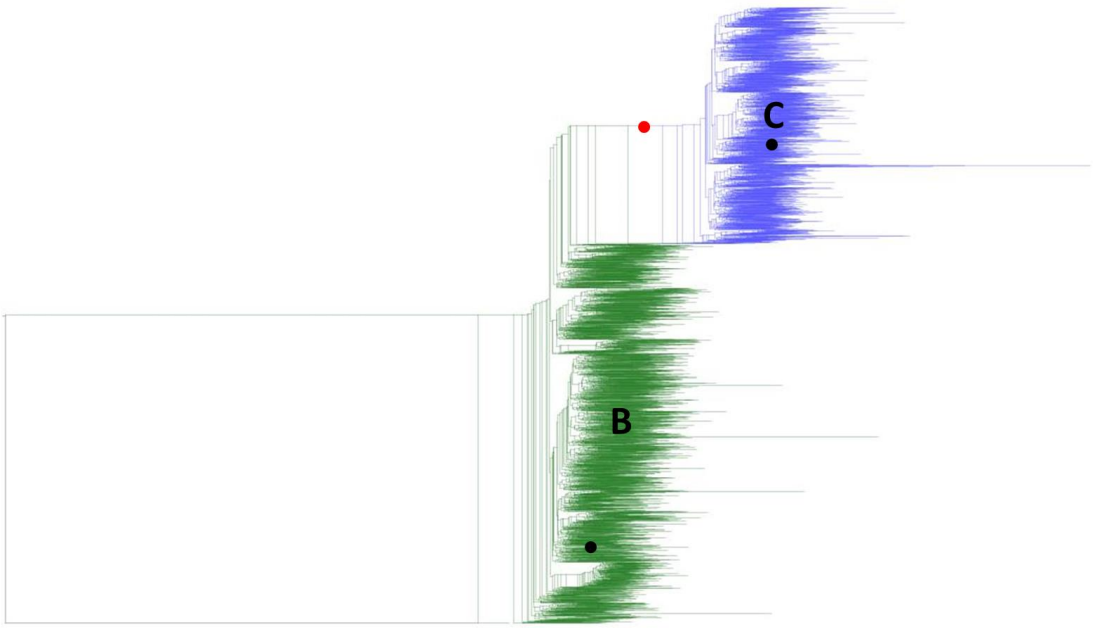
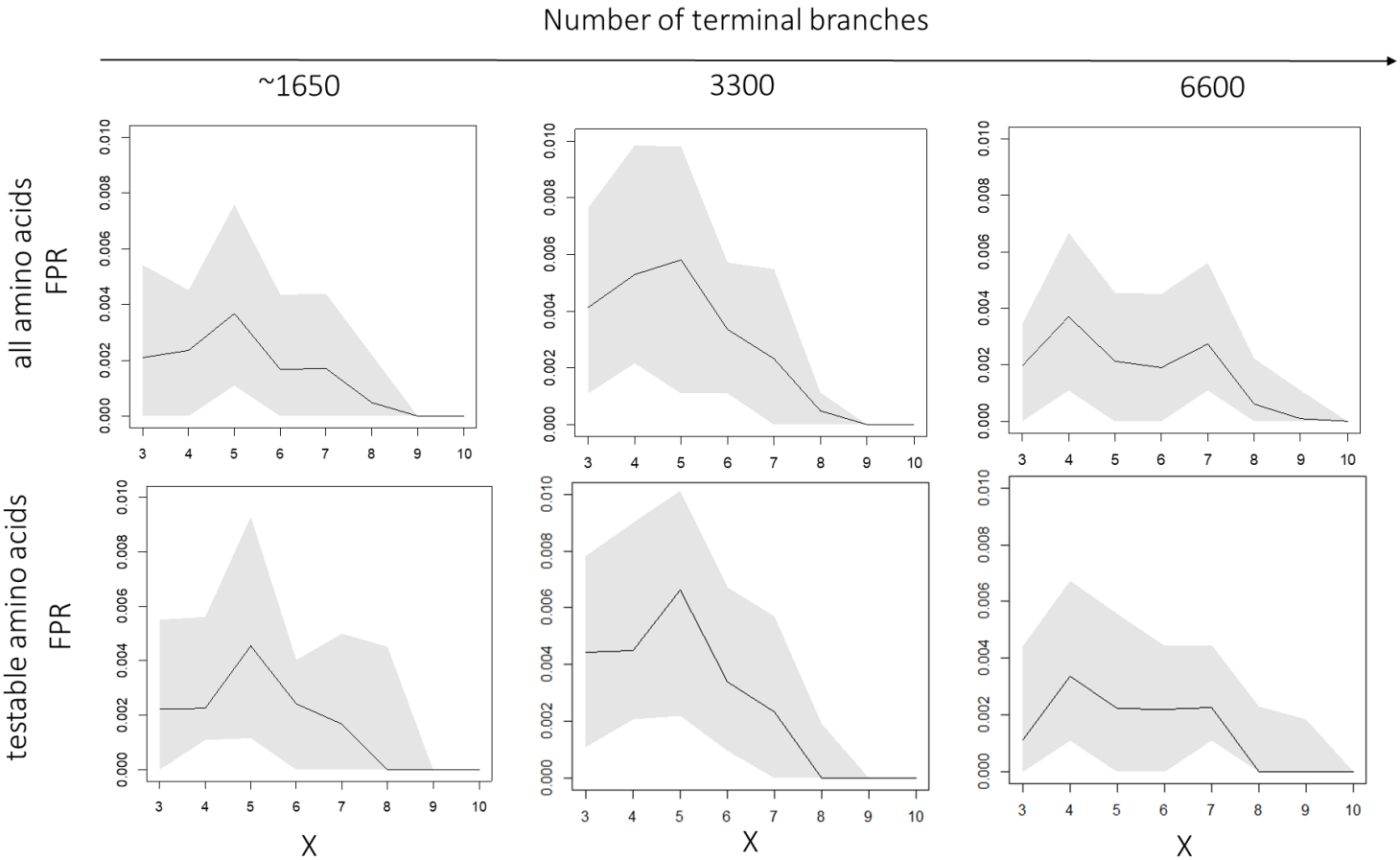
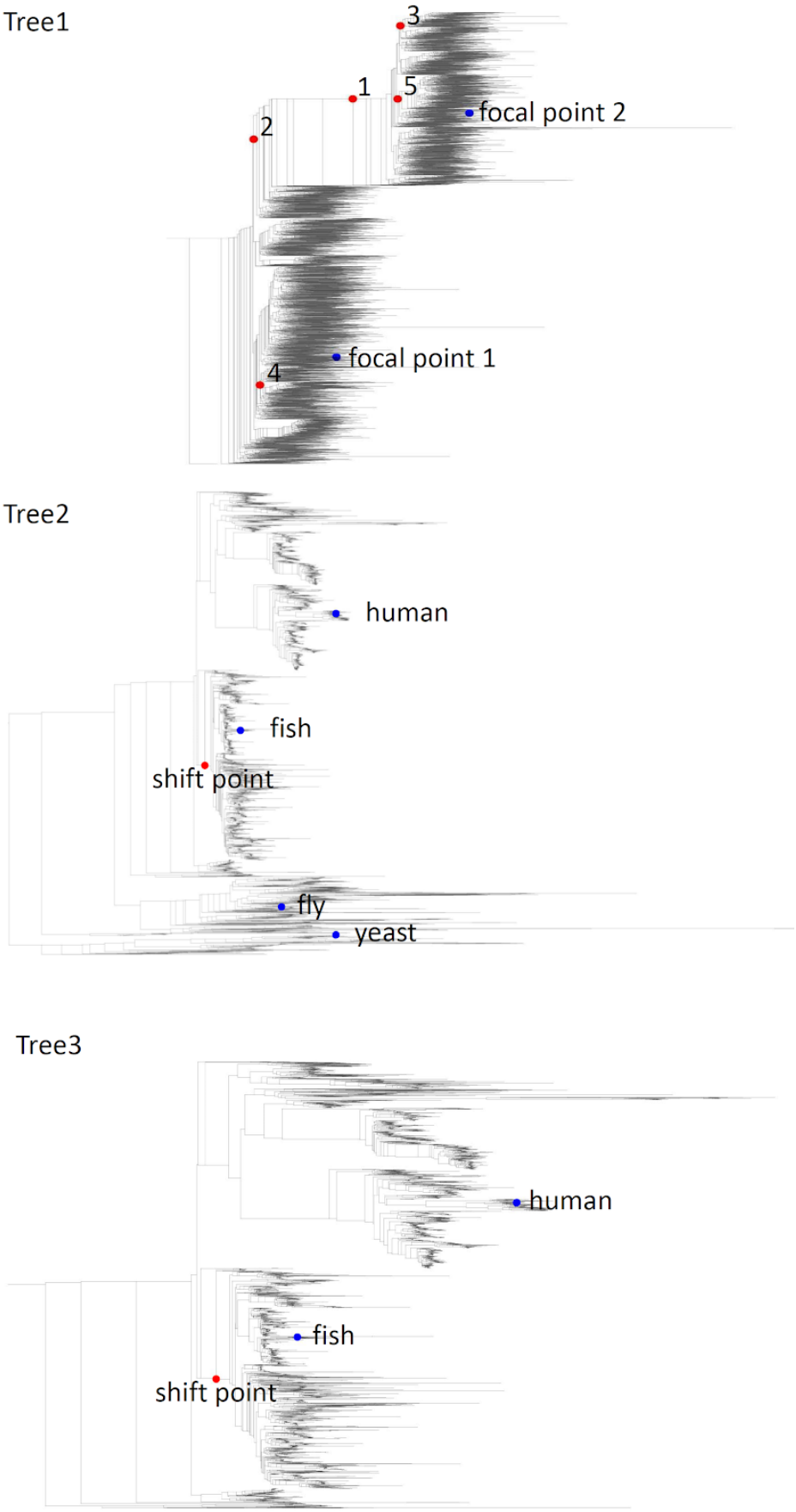
Supporting Information



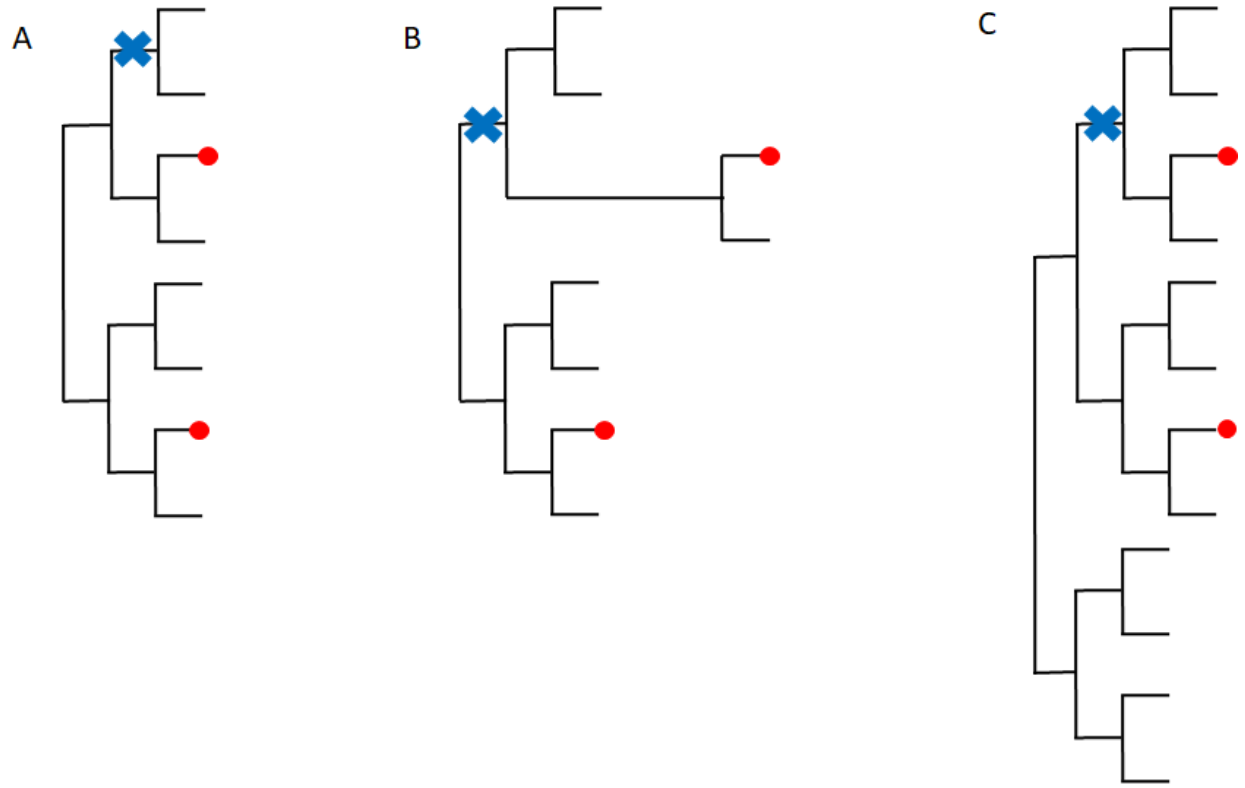
Supplementary figure 1. Phylogenetic tree of B and C HIV-1 subtypes used in simulation analysis. Green clade is subtype B; blue clade is subtype C; black dots are the two strains to which we applied our test in simulation analysis; red dot is a point of fitness shift.



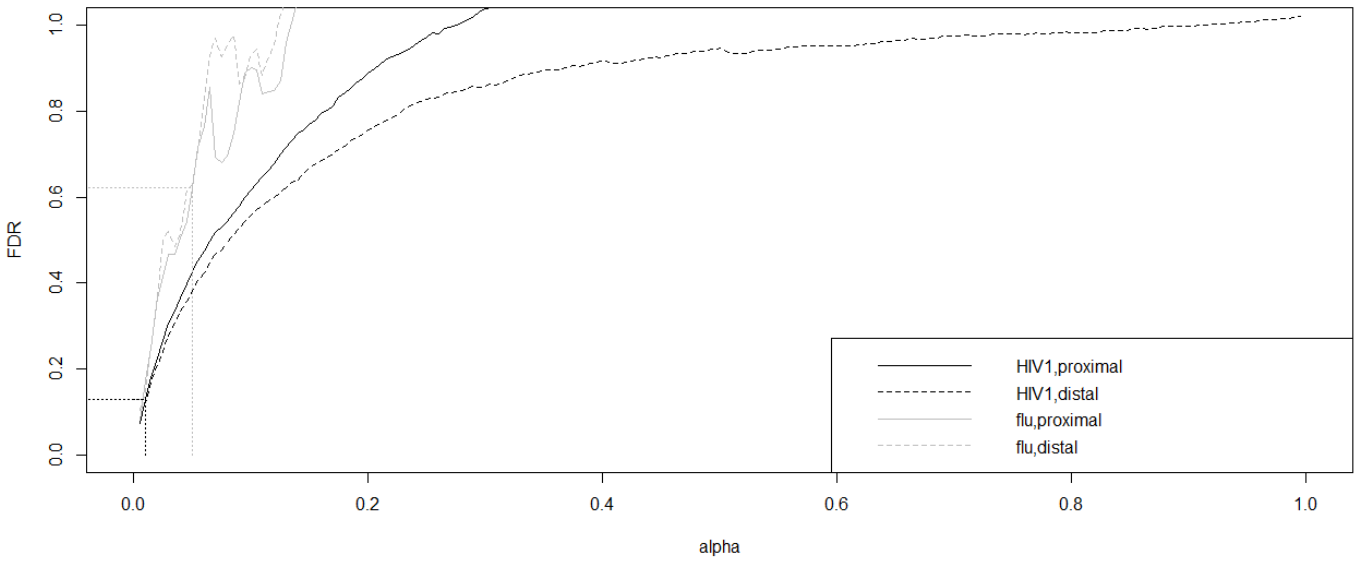
Supplementary figure 2. False Positive Rate of our approach for phylogenetic trees with ~1650, 3300 and 6600 species in simulations for different scaled fitness values of the preferred variant *X;* black line, mean; grey area, 90% confidence interval from random sampling of 1000 amino acids for 100 times. Upper row: all amino acids are considered, bottom row: only testable amino acids are considered.



Supplementary Figure 3. Various conditions for simulations described in Supplementary Table 2. Blue dots - focal nodes, red dots - alternative points of shift in fitness vectors.



Supplementary Figure 4. Schematic representation of cases that can decrease performance of our test. Red dots, focal nodes; blue cross, point of fitness shift. A, both focal points have the same fitness vector, but fitness shift occurs closer to one of them, leading to false positives; B, there is a long branch between the fitness shift and one of the focal points, leading to false negatives; C, distance between the two focal points is less than mean distance between one of them and species with the same fitness landscape, leading to false negatives.



Supplementary figure 5. Q-value for different significance thresholds obtained as min (α*P)/V, where α is the significance threshold, P is the number of testable amino acids at this α, and V is the number of significant tests at this α, in testing of the hypothesis that an amino acid is proximal or distal for one focal node. Black, gp160; grey, HA; solid, hypothesis of proximity; dashed, hypothesis of distality.

Dotted lines correspond to the significance threshold used in the main analysis for gp160 (black) and HA (grey).

Supplementary table 1. Power of d-test in simulations with different selective constraints

	TPR			FPR		
X / tree	4-9 (mean)	3	10	4-9 (mean)	3	10
all amino acids						
huge	0.75	0.41	0.24	0.002	0.001	0
middle	0.43	0.29	0.1	0.003	0.004	0
cutted	0.18	0.09	0.02	0.001	0.002	0
only testable amino acids						
huge	0.77	0.4	0.48	0.002	0.001	0
middle	0.49	0.29	0.56	0.003	0.004	0
cutted	0.28	0.09	0.4	0.001	0.002	0

Supplementary table 2. Performance of the d-test in different conditions. Phylogenetic trees with focal nodes and points of fitness shift are shown in Supplementary Figure 3. * - cases when fitness shift occurred in a phylogenetic neighborhood of one focal node, but both focal nodes had the same fitness vector. Fitness of amino acids with changing preferences is shown in red. Sensitivity and specificity that maximized Yoden's coefficient (TPR-FPR) and therefore may serve as the "optimal" performance are shown in "max(Yoden's coeff)" column. Only testable amino acids were used to calculate the performance.

Tree1						
simulations with fitness shift						
FitnessVector1	FitnessVector2	ShiftPoint	α=0.01		max(Yoden's coeff)	
			sensitivity	specificity	sensitivity	specificity
8111…1	1811…1	1	0.58	0.9993	0.85	0.95
8811…1	1188…1	1	0.42	0.9946	0.75	0.8
8811…1	8111…1	1	1	0.864	1	0.9
8811…1	8181…1	1	0.97	0.992	0.99	0.98
812345671…1	182345671…1	1	0.76	0.992	0.9	0.9
8812345671…1	8182345671…1	1	0.74	0.995	0.87	0.96
8 1 1.1 1.2…1.7 1…1	1 8 1.1 1.2…1.7 1…1	1	0.61	0.997	0.87	0.94
8111…1	1811…1	2	0.07	1	0.39	0.89
8111…1	1811…1	3*	-	0.91	-	-
8111…1	1811…1	4	0.6	0.99	0.83	0.92
8111…1	1811…1	5	0.79	0.994	0.86	0.97
simulations with constant fitness						
8111…1	-	-	-	0.9991	-	-
8811…1	-	-	-	1	-	-
812345671…1	-	-	-	0.9993	-	-

8812345671…1	-	-	-	0.9972	-	-
8 1 1.1 1.2…1.7 1…1	-	-	-	0.997	-	-
Tree2						
FitnessVector1	FitnessVector2	FocalNodes	question1			
			α=0.01		max(Joden's coeff)	
			sensitivity	specificity	sensitivity	specificity
8111…1	1811…1	human-yeast*	-	0.996	-	-
8111…1	1811…1	fish-yeast	0.02	0.997	0.15	0.94
8111…1	1811…1	fish-fly	0.04	1	0.25	0.96
8111…1	1811…1	fish-human	0	1	0.04	0.99
Tree3 (part of Tree2 with root in the lowest common ancestor of vertebrates)						
8111…1	1811…1	fish-human	0.2	1	0.46	0.94

Supplementary table 3. Some of variable fitness amino acids are located in sites with different antigenicity in viral subtypes.

antibody	epitope	PDB id	number of positions in the epitope*	of them, sites with variable fitness amino acids for subtypes A and B	of them, sites with variable fitness amino acids for subtypes B and C	Wilcoxon two-sided test for IC50 in subtypes A and B	Wilcoxon two-sided test for IC50 in subtypes B and C
VRC01	CD4-binding site	3NGB	42	2	13	9.39E-02	0.2325
PG9	V1/V2 loop	3U4E	13	1	2	**3.00E-02****	**0.003902**
VRC01	CD4-binding site	3NGB	42	2	13	**0.004878**	**0.01023**
NIH45-46		4JKP	n/a	n/a	n/a	0.826	0.05439
b12		2NY7	54	6	15	**0.000195**	**1.71E-03**
PG9	V1/V2 loop	3U4E	13	1	2	**1.70E-04**	**0.0003272**
2G12	glycans in the outer domain of gp120	6E5P	5	0	2	**2.62E-02**	**4.42E-08**
2F5	gp41	n/a	n/a	n/a	n/a	0.9361	**7.76E-05**
4E10		n/a	n/a	n/a	n/a	0.2329	0.7788
PGT121	outer domain of gp120 and the V3 loop base	5CEZ	20	0	2	0.826	0.9348
PGT128		5C7K	21	0	2	0.3986	0.07562

*According to [35]

p-values less than 0.05 are shown in **bold

Supplementary table 4. Consensus amino acids in a pair of viral subtypes in sites with variable fitness amino acids that reside in epitopes of antibodies with different binding capacity in two subtypes.

A-B comparison

antibody	higher affinity to subtype	site (hxb2 numbering)	amino acid in A strain	amino acid in B strain
VRC01	A	281	A	A
VRC01	A	425	N	N
b12	B	178	R	K
b12	B	181	I	V
b12	B	281	A	A
b12	B	425	N	N
b12	B	426	M	M
b12	B	430	V	V
PG9	A	168	K	K

B-C comparison

antibody	higher affinity to subtype	site (hxb2 numbering)	amino acid in B strain	amino acid in C strain
b12	B	178	K	R
b12	B	179	L	L
b12	B	181	V	I
b12	B	182	V	V
b12	B	183	P	P
b12	B	192	R	R
b12	B	198	T	T
b12	B	281	A	A
b12	B	384	Y	Y
b12	B	386	N	N
b12	B	421	K	K
b12	B	426	M	M
b12	B	429	E	E
b12	B	455	T	T

b12	B	471	G	G
PG9	C	160	N	N
PG9	C	168	K	K
2G12	B	332	N	N
2G12	B	339	N	N