Correlated Evolution of Nearby Residues in Drosophilid Proteins

Benjamin Callahan¹*, Richard A. Neher²*, Doris Bachtrog³, Peter Andolfatto⁴, Boris I. Shraiman²,⁵

¹ Department of Applied Physics, Stanford University, Stanford, California, United States of America, ² Kavli Institute for Theoretical Physics, University of California Santa Barbara, Santa Barbara, California, United States of America, ³ Department of Integrative Biology and Center for Theoretical Evolutionary Genomics, University of California Berkeley, Berkeley, California, United States of America, ⁴ Department of Ecology and Evolutionary Biology and the Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, New Jersey, United States of America, ⁵ Department of Physics, University of California Santa Barbara, Santa Barbara, California, United States of America

Abstract

Here we investigate the correlations between coding sequence substitutions as a function of their separation along the protein sequence. We consider both substitutions between the reference genomes of several Drosophilids as well as polymorphisms in a population sample of Zimbabwean Drosophila melanogaster. We find that amino acid substitutions are “clustered” along the protein sequence, that is, the frequency of additional substitutions is strongly enhanced within ≈10 residues of a first such substitution. No such clustering is observed for synonymous substitutions, supporting a “correlation length” associated with selection on proteins as the causative mechanism. Clustering is stronger between substitutions that arose in the same lineage than it is between substitutions that arose in different lineages. We consider several possible origins of clustering, concluding that epistasis (interactions between amino acids within a protein that affect function) and positional heterogeneity in the strength of purifying selection are primarily responsible. The role of epistasis is directly supported by the tendency of nearby substitutions that arose on the same lineage to preserve the total charge of the residues within the correlation length and by the preferential cosegregation of neighboring derived alleles in our population sample. We interpret the observed length scale of clustering as a statistical reflection of the functional locality (or modularity) of proteins: amino acids that are near each other on the protein backbone are more likely to contribute to, and collaborate toward, a common subfunction.

Introduction

There has been an ongoing debate over the past few decades about the processes underlying protein evolution [1–5]. The neutral theory [1] posits that protein evolution is chiefly governed by the fraction of newly arising mutations that are not detrimental enough to be removed by natural selection. However, recent population genetic analyses of closely related Drosophila species suggest that protein divergence between species is substantially in excess of the neutral model’s predictions [6,7]. Intriguingly, this protein divergence excess is consistent with an important role for the contribution of weakly deleterious mutations to this pattern is still debated [10,11].

The dramatic shift in our view of the processes driving protein evolution in Drosophila highlights the deficiency in our understanding of the mechanisms responsible for the observed protein divergence excess. One reason for this deficiency is the explicitly sequence-based nature of the population genetic analyses used to describe the excess divergence. These methods were developed for the analysis of linear sequences of independently evolving amino acids, and quite generally ignore the fact that most proteins fold into complex three-dimensional structures, held together by interactions between amino acids and between amino acids and the surrounding medium. Protein function depends critically on this folded structure, e.g. the arrangement of specific amino acids at the active site of an enzyme [12]. This is reflected in protein evolution; both the structure and the function of homologous proteins are remarkably conserved over long times, even while primary sequences substantially diverge [13]. The maintenance of protein structure is possible because evolution preserves structurally important interactions, such as favorable biochemical interactions between amino acids in physical contact [14]. This preservation of structurally important interactions affects sequence-based analyses; the preferred state and variability of an amino acid will depend on amino acids elsewhere in the protein [15].

This study is motivated by the desire to more closely integrate protein structure and function into sequence-based inferences of selection. Correlations between substitution patterns and protein structure have yielded insights over many years, from the slower divergence of protein active sites [1,16] to recent results indicating a correlation between estimates of positive selection and secondary structure [17]. Work demonstrating the evolutionary consequences...
of interactions inferred from RNA structure [18–20] supported the application of sequence-based inference of functional interactions to proteins, where functional interactions are difficult to identify even when structure is known [21]. Under the assumption that functionally interacting residues coevolve, interactions can be identified if enough evolutionary trajectories can be sampled. In practice this has meant multi-alignments across many species of large protein families [22–25], but alignments within populations of the highly mutable HIV have also been used [26,27]. These methods have been successfully used to identify pair-wise interactions between residues that contribute to protein function. As an example, the inclusion of interactions inferred from a multi-alignment was shown sufficient to produce a stable fold [28].

Here we develop a complementary approach intended to probe the level of influence interactions have on protein evolution. Instead of focusing on a single protein and specific pairs of interacting residues, we shall aggregate evolutionary information across proteins and use the increased statistical power to look for generic patterns. Specifically, we investigate the correlations in the substitution processes at residues a given distance from each other along the protein backbone, averaged over many proteins of D. melanogaster. Our rationale is as follows: residues that are near in the primary protein sequence are also likely to be near in the folded protein (Figure 1A) and therefore more likely to interact physically and/or belong to the same protein domain. Consequently, if correlated evolution in proteins is common, it should be detectable by an increase in evolutionary correlation between residues nearby in sequence, for which physical interaction in the folded protein is more likely. While we will be unable to identify particular interactions, our approach will be informative about the overall level of influence interactions have on the evolution of proteins.

We find that amino acid substitutions cluster together on the protein sequence, i.e. amino acid substitutions are more frequent nearby other such substitutions. The strength of this effect decays exponentially with the separation between the residues along the protein sequence, with a characteristic length scale of about 10 codons. We observe this clustering phenomenon in substitutions between D. melanogaster and several sister species (Figure 1B) as well as in polymorphisms within a Zimbabwean population sample of D. melanogaster. Clustering is absent when considering synonymous substitutions, implicating selection as the root cause. Furthermore, clustering is stronger between substitutions that arose along the same branch of the evolutionary tree than between substitutions that arose in different branches, and nearby derived alleles tend to cosegregate in our population sample. Additionally, pairs of substitutions within 10 codons of each other that arose in the same lineage have a significant tendency to cause compensatory changes to the total charge of the protein. These lines of evidence lead us to conclude that epistasis between amino acid substitutions contributes significantly to clustering, and the substitution process as a whole.

Results

The 12 Drosophilid genomes resource [29] serves as the primary data source in this study. We used this resource to identify protein coding sequence substitutions between D. melanogaster (Dmel) and several sister Drosophilids: D. sechellia (Dsec), D. simulans (Dsim), D. yakuba (Dyak), D. erecta (Dere), D. ananassae (Dana) and D. pseudoobscura (Dpse) available at http://rana.lbl.gov/ drosophila/ (Figure 1B). Substitutions were ascertained from nucleotide alignments of the reference genomes produced by the blastz algorithm [30], and available from UCSC [31] at ftp://hgdownload.cse.ucsc.edu/goldenPath/dm3/.

Our goal here is to understand how correlation between the substitution processes at different residues is affected by the distance between those residues along the protein sequence. To this end we introduce the conditional probability function (cPDF), which we denote $C_f(y)$ and define as the probability of there being a substitution of type $f$ at sequence position $x+y$ conditioned on the existence of a substitution of type $f$ at sequence position $x$. To assess, for example, whether the probability of a synonymous divergence (DS) is affected by the presence of a non-synonymous divergence (DN) some distance $y$ away, we can estimate $C_{DN}^{DS}(y)$ and compare it to the overall level of synonymous divergence.

cPDFs are estimated from sets of aligned coding sequences by averaging over all instances of the focal substitution $f$ in the aligned sequences (Methods). Since we are particularly interested in the functional dependence of cPDFs on the distance from the focal substitution $y$ we will normalize cPDFs by their asymptotic value (Methods). Note that we will always be measuring distance $y$ in terms of codons rather than nucleotides, as this is the natural unit of distance in a gene. Figure 2A shows three of these normalized cPDFs, $C_{DN}^{DN}(y)$, $C_{DN}^{DS}(y)$, and $C_{DN}^{DS}(y)$, estimated from the species comparison of D. melanogaster and D. yakuba.

Amino acid substitutions cluster along protein sequences

Amino acid substitutions are not distributed uniformly along the protein sequence. The cPDF for non-synonymous substitutions, $C_{DN}^{DS}(y)$, is significantly peaked around $y=0$ in every species comparison we consider. This peak describes the tendency of non-synonymous substitutions to ‘clump together’ on the protein sequence, a phenomenon we call clustering. The shape of the clustering peak is well-fit by a decaying exponential with a characteristic length scale of about 10 codons. In sharp contrast, the cPDFs involving synonymous substitutions, $C_{DN}^{DS}(y)$ and $C_{DN}^{DS}(y)$, have no clustering peak, indicating that synonymous substitutions are distributed uniformly along the protein sequence. The difference between non-synonymous and synonymous
clustering is highly significant, the sampling p-value is essentially zero \( p < e^{-100}, \) chi-square test.

The magnitude of clustering is large. The nearest neighbor of a codon with a non-synonymous substitution is roughly twice as likely to also have such a substitution than would otherwise be expected. The impact of clustering extends well beyond the nearest neighbor, and is appreciable out to a distance of at least 20 codons from a focal non-synonymous substitution. We quantify the total magnitude of clustering by defining the ‘clustering count’ \( \Omega_f \) as the difference between the expected number of substitutions of type \( f \) in the 20 codons downstream of a focal substitution of type \( f \) and the expected number in a 20 codon sequence segment distant from the focal substitution (Methods). More plainly, \( \Omega_f \) is the number of extra \( f \) substitutions you find in the vicinity of an \( f \) substitution because substitutions cluster instead of being distributed uniformly along the sequence. Graphically, \( \Omega_f \) is the area

### Figure 1. Structural distance as a function of sequence distance and Drosophilid phylogeny.

A) All PDBs for the source organism \( D. \) melanogaster were downloaded at http://www.pdb.org/, with homologs excluded at 90% identity. These crystal structures were used to estimate a relationship between the structural distance between the \( C_\alpha \) atoms of amino acids as a function of their separation along the primary sequence. The solid blue line indicates the mean distance, while the dashed lines indicate first and third quartiles. Structural distance increases quickly with sequence distance, but the increase saturates at a sequence distance of around 10 amino acids. B) The phylogenetic tree of the Drosophilid species we are considering (adapted from that at http://rana.lbl.gov/drosophila/).

doi:10.1371/journal.pgen.1001315.g001

### Figure 2. The frequency of additional substitutions near a focal substitution.

\( CDN(\gamma) \) is the conditional probability of finding a non-synonymous divergence (DN) a distance \( \gamma \) from another DN, relative to the unconditional probability. The peak of \( CDN(\gamma) \) around \( \gamma \approx 0 \) describes the enhanced frequency of DNs near other DNs over a length scale of approximately 10 codons, an effect we call local ‘clustering’. There is no clustering when synonymous substitutions (DS) are considered. Raw data is plotted here as crosses, the solid lines are moving window smoothings (Methods). A) The cPDFs estimated from the set of aligned coding sequences for the species comparison of \( D. \) melanogaster and \( D. \) yakuba are shown. The range of the cPDFs estimated from all other species comparisons in this study is indicated with solid background colors, demonstrating the consistency of this signal. Figure S1 displays the amount of coding sequence included for each species comparison. B) The special case in which diverged codons are separated by an intron (minimum length of 44 nt). The estimated cPDFs are noisier at low \( \gamma \) because nearby sites are unlikely to span an intron, but the clustering peak of \( CDN(\gamma) \) is clearly consistent with that found within exons (shown with the dashed line), particularly the length scale. C) The cPDFs between polymorphisms, both synonymous (PS) and non-synonymous (PN), found in 130 kb of coding sequence (182 genes) in a Zimbabwean population sample of \( D. \) melanogaster. Polymorphisms cluster analogously to substitutions.

doi:10.1371/journal.pgen.1001315.g002
Amino Acid Changes Cluster in Protein Sequence

under the clustering peak (and above the asymptotic value) of the normalized cPDF $C'_{DN} (y)$, multiplied by the overall density of $J'$ substitutions.

We are particularly interested in $\Omega_{DN}^{\text{CDN}}$, which we will simply denote $\Omega$. The shape of $C_{DN}^{\text{CDN}} (y)$ is very consistent between the different species comparisons tested, but the clustering count $\Omega$ is not because it depends not only on $C_{DN}^{\text{CDN}} (y)$, but also on the density of substitutions between the species being compared. $\Omega$ ranges from 0.26 in the D. melanogaster versus D. sechellia alignment to 0.68 in the D. melanogaster versus D. ananassae alignment, as seen in Figure 3A. $\Omega$ increases linearly with $D_n$ (the fraction of substituted amino acids), this is consistent with a clustering pattern that remains constant as divergence increases with time.

Clustering between nearby non-synonymous substitutions is strongly supported by the data, but it is not a priori clear whether it is the separation of amino acids along the protein backbone, or the distance in base pairs along the genome, that matters. To discriminate between these possibilities we repeated the correlation analysis including only those pairs of residues which spanned an intron. As a result the genomic separation between codons had a median increase of 70 bp (~23 codons) and a minimum increase of 44 bp (~15 codons), while separation between the encoded amino acids along the protein backbone was unchanged. As shown in Figure 2B, the cPDFs estimated from these intron-spanning pairs of codons correspond closely with those estimated within exons, when separation along the protein backbone (exonic distance) is used in the estimation. We conclude that the clustering length scale is set by the distance along the protein backbone, not along the genome.

Remarkably, the clustering between amino acid substitutions is not limited to substitutions between species. It is also apparent among polymorphisms within a population sample of D. melanogaster (Methods). Figure 2C shows the estimated cPDFs between synonymous and non-synonymous polymorphisms ($PN$ and $PS$). The cPDFs estimated from polymorphisms are much noisier because our population sample sequencing spans only 130 kb of coding sequence, as compared to ~15 Mb for the divergence data. Nevertheless, we find clustering between polymorphisms analogous to that between substitutions: non-synonymous polymorphisms cluster significantly ($p = 1.4 \times 10^{-8}$, chi-square test), while synonymous polymorphisms do not.

Factors influencing clustering

We tested for potential relationships between clustering and a number of genetic properties by estimating $\Omega$ on subsets of the full set of coding sequences stratified by the property in question. Clustering is robust in the sense that it is not substantially affected by many of the properties we tested, such as chromosome (including autosome versus X), recombination rate and the level of gapping in the alignment (Figures S2, S3, S4). We did find a systematic relationship between the GC content of coding sequence and clustering; higher GC content correlates with stronger clustering (Figure S5).

A notable factor that influences clustering is the level of constraint under which a gene evolves, which we estimate by the fraction of substituted amino acids $D_n$. Amino acid substitution are more clustered in constrained genes than they are in unconstrained genes, i.e. $C_{DN}^{\text{CDN}} (y)$ has a larger clustering peak when it is estimated from highly constrained (low $D_n$) coding sequences, see Figure 3B. In the inset of Figure 3B we have plotted the $\Omega$ estimated from each subset of coding sequences against the average $D_n$ of that subset. It is useful to compare this plot to the one in Figure 3A, which also is a plot of $\Omega$ versus $D_n$. The difference between these plots is that in panel A $D_n$ effectively measures divergence time and $\Omega$ scales linearly with $D_n$, while in the inset of panel B $D_n$ tracks the level of constraint and $\Omega$ is

![Figure 3](https://example.com/figure3.png)

**Figure 3. The dependence of clustering count $\Omega$ on sequence divergence.** A) The number of additional amino acid substitutions expected in the vicinity of a focal substitution due to clustering, $\Omega$, increases linearly with divergence between species. This is seen in this plot of $\Omega$ against the fraction of substituted amino acids $D_n$ for six comparisons of D. melanogaster to sister Drosophilids. B) Non-synonymous substitutions cluster more strongly in more constrained genes. Here $C_{DN}^{\text{CDN}} (y)$ is estimated from subsets of the aligned coding sequences for the species comparison of D. melanogaster and D. yakuba. The subsets correspond to the ten deciles of the coding sequences ranked by non-synonymous divergence. More constrained genes (lower $D_n$, darker color) have more pronounced clustering, seen as the larger peak of $C_{DN}^{\text{CDN}} (y)$ near $y = 0$. The inset shows clustering count $\Omega$ versus the average $D_n$ of each subset. $\Omega$ increases linearly at low $D_n$, but quickly levels off and is roughly constant at ~0.5 for $D_n > 0.05$. This contrasts with the result in panel A where $D_n$ measured divergence time, rather than constraint, and $\Omega$ increased linearly with $D_n$.

doi:10.1371/journal.pgen.1001315.g003
strongly sublinear in $D_n$. In fact, once constraint relaxes past a certain point, $\Omega$ becomes roughly constant. This relationship suggests that substitutions in constrained genes occur in tight clusters, and that as constraint lessens the additional substitutions which accrue do so uniformly along the sequence.

Potential non-selective causes of clustering

Clustering of amino acid substitutions is due to selection

Non-selective mechanisms cannot account for both significant non-synonymous clustering and the absence of synonymous clustering. Having ruled out non-selective mechanisms, we now consider potential selective mechanisms that could cause amino acid substitutions to cluster. Perhaps the simplest explanation for clustering is that proteins have short segments, such as unstructured loops, that are under reduced purifying selection. These weakly constrained segments experience locally increased rates of amino acid substitution, which we then observe as clustering in both divergence and polymorphism data. There are also several ways in which positive selection could cause clustering. Clustering could be the result of localized ‘adaptive bursts’, i.e. functional modules in which multiple independently adaptive substitutions became available (perhaps due to a changed environment). Because amino acids close on the protein backbone are more likely to be in the same module, the resulting burst of adaptive substitutions would be clustered on the sequence. Amino acids that are close along the chain are also more likely to physically interact, even after protein folding. As a consequence, the fitness effect, and hence evolutionary fate, of nearby substitutions could be contingent on one another (i.e. epistasis). In particular we might imagine common compensatory interactions between nearby substitutions, although all synergistic interactions would contribute to clustering. Finally, another potential mechanism is hitchhiking. In this scenario mildly deleterious amino acid polymorphisms are driven to fixation by the selective sweep of a linked allele, resulting in clustered substitutions. We will now attempt to disentangle the relative contributions of these different selective scenarios.

Clustering of amino acid substitutions tends to occur in the same lineage

We can polarize substitutions by the lineage on which they arose using an outgroup and then repeat our correlation analysis for pairs of substitutions which arose in the same lineage and for pairs which arose in different lineages (Methods). This allows us to begin to distinguish between potential selective mechanisms of clustering. If spatial heterogeneity in the strength of purifying selection is responsible for clustering we expect equal clustering within and between lineages, since in this case the presence of a substitution simply informs as to the level of constraint in that region of the protein sequence. In contrast, the alternative selective mechanisms (adaptive bursts, compensatory or synergistic mutations, and hitchhiking) are lineage-specific, they only apply when substitutions occur in the same lineage and therefore can only cause clustering between same-lineage substitutions.
We incorporate polarization into our analysis by extending the sequence features f in our cPDFs with the specification of the species lineage on which a substitution arose, e.g. f = Dvla is a non-synonymous substitution in the a ∈ {Dmel, Dsec, Dsim, Dyak, Dere, Dana, Dpse} lineage (Methods). The non-synonymous cPDFs estimated for substitutions in the same and different lineage than the focal substitution are shown in Figure 4A for each species comparison. Clustering between those substitutions is always significant whether substitutions arose in the same lineage or in different lineages, but clustering between same lineage substitutions is always significantly stronger (Table S1). We argued above that spatially heterogeneous purifying selection would cause equal clustering within and between lineages. If this is so, the excess clustering within lineages must be generated by one of the lineage-specific alternatives.

Excess lineage-specific clustering can be quantified with an extension of the clustering count \( \Omega(a) \). First we define the lineage-specific clustering count \( \Omega(a) \) as an analog of \( \Omega \) with the difference that the cPDF from which \( \Omega(a) \) derives is estimated using only substitutions in lineage a. Therefore, \( \Omega(a) \) is the increased number of a-lineage DNs near a focal a-lineage DN due to clustering. Next, the ‘lineage-specific excess clustering count’ \( \Delta \Omega(a) \) is the portion of \( \Omega(a) \) which is inconsistent with a lineage non-specific mechanism. We quantify this as the difference between the within-a and between-lineage clustering over the first 20 codons (Methods). This corresponds graphically to the area between those cPDFs (the red area in Figure 4A), \( \sum_{x=1}^{20} \left( C_{DN}\text{x}(y) - C_{DN}\text{y}(y) \right) \), multiplied by the density of substitutions in the lineage a.

The lineage-specific excess \( \Delta \Omega(a) \) appears to be a roughly constant fraction of the total lineage-specific clustering \( \Omega(a) \). The estimate of \( \Delta \Omega(a) \) is plotted against the estimate of \( \Omega(a) \) for both lineages of all our species comparisons in Figure 4B. This relationship is well-fit by a linear model, suggesting that approximately 1/3 of clustering within a lineage is due to lineage-specific mechanisms, i.e. some combination of compensatory or synergistic mutations, adaptive bursts and hitchhiking. The D. simulans lineage is an outlier, \( \Delta \Omega(D\text{sim}) \) is aberrantly high. This may be a consequence of details relating to this particular reference sequence: the D. simulans reference sequence has lower coverage and quality than the other reference sequences as well as being a ‘mosaic’ assembly constructed from multiple individuals [29]. The D. melanogaster lineage is also picked out by the synonymous control, there is significant synonymous clustering in this lineage above that found in any other lineage we consider (Figure S10).

**Nearby charge-altering substitutions tend to compensate each other**

If compensatory mutations are contributing substantially to lineage-specific excess one might find evidence of this in a physical or biochemical quantity associated with the compensation. For example, changes in volume, hydrophobicity, charge, etc. might anti-correlate if the substitutions are compensatory. We tested several amino acid properties for such a relationship but found only one that exhibited the hypothesized behavior: nearby substitutions have a significantly increased probability to cause compensatory changes in charge, but only when they arise in the same lineage! We quantify this effect by estimating the fraction of substitutions which compensate the effect of a focal charge-altering substitution, as a function of distance from the focal substitution y. In Figure 5 we see that the fraction of charge-compensating
substitutions is significantly elevated near a focal charge-altering substitution, on roughly the clustering length scale of 10 codons. This compensation serves to partially conserve the total charge of the protein sequence within the clustering length scale.

Local charge compensation is significant in every species comparison we considered, all p-values <10^{-17}, chi-square test (Table S2). A measure of the magnitude of this effect is the fraction of charge-altering substitutions that have their charge alteration compensated for by the net change in charge caused by the other substitutions within 10 codons. This varies by lineage, but is always significant and increases with species divergence up to 15% for the species comparison of D. melanogaster and D. pseudoobscura. Charge compensation is a lineage-specific effect, and it is responsible for a significant fraction of the lineage-specific excess ΔΩ(x) we observe, roughly 5—10% depending on lineage (Table S2). The observation of substantial charge compensation, and the lack of compensation of other amino acid properties, is consistent with previous observations which suggested charge compensation to be of greater significance in protein evolution than compensation of other amino acid characteristics [22,34]. Interestingly, while substitutions in different lineages do not exhibit the local compensation phenomenon, they do show a weaker, but statistically significant, increase in the fraction of nearby changes which alter charge in the same direction, perhaps indicating convergent evolution (Table S2).

Figure 5. Amino acid substitutions tend to conserve local charge. Several amino acids are charged, with charges equal to ±1. Consequently, an amino acid substitution can cause a change in protein charge Δc ∈ {−2, −1, 0, 1, +2}. Conditioned on a focal charge-altering substitution (Δc(x) ≠ 0), we ask whether nearby substitutions tend to reinforce or compensate the focal charge change in charge, i.e. whether Δc(y) ≠ 0 and has the same sign as Δc(x) (reinforcing) or the opposite sign (compensating). The fraction of substitutions which compensate/reinforce the focal charge-altering substitution is plotted as a function of the separation between the sites y=±x, estimated from the species comparison D. melanogaster and D. yakuba. Distinction is made between substitutions which arose in the same and different lineage as the focal substitution. Substitutions in the same lineage tend to be compensatory when within the clustering length scale of 10 codons (p=5.63×10^{-3}, chi-square test). This is not observed for substitutions that arose in a different lineage, in that case nearby substitutions are more likely to alter charge in the same direction.

doi:10.1371/journal.pgen.1001315.g005

Nearby amino acid mutations cosegregate in a population

Non-synonymous polymorphisms cluster as well, and polymorphism data provides another avenue to distinguish between the possible selective mechanisms of clustering. Under a model of bursts of independent adaptive mutations, beneficial amino acid mutations can be incorporated sequentially, and would not be expected to segregate together in the population since beneficial mutations rapidly fix after arising. In contrast, if epistatic selection is driving the observed clustering we expect that a compensatory mutation will only be found on a chromosome that already carries the first mutation, i.e. we expect the derived states of nearby polymorphic sites to cosegregate. We can quantify this expectation by estimating the average polarized linkage disequilibrium ⟨D_{mu}(y)⟩ [35,36], i.e. the frequency of the doubly derived haplotype minus the product of the frequencies of the individual derived alleles averaged over all pairs of polymorphisms a distance y apart. ⟨D_{mu}⟩>0 then indicates that derived alleles occur in coupling more often than would be expected if their fitnesses were independent. Consistent with the compensatory scenario, we find ⟨D_{mu}⟩>0 when estimated from amino acid polymorphisms within 5 codons of each other, as seen in Figure 6.

We evaluate the significance of the cosegregation of nearby derived alleles by bootstrapping: we resample polymorphic sites from the full set of polymorphic sites in our population, pair them off into a number of pairs equal to the number of pairs of polymorphisms within 5 codons of each other, and then estimate ⟨D_{mu}⟩ from this resampled ensemble. Repeating this process 10^7 times yields a bootstrapped probability distribution p_{boot}(⟨D_{mu}⟩) which we compare to the ⟨D_{mu}⟩ estimated from the data, yielding a bootstrapping p-value of p=2×10^{-4} of observing an equal or greater ⟨D_{mu}⟩ by chance from our population sample. Again, only pairs of non-synonymous polymorphisms significantly cosegregate, supporting the contention that epistasis is responsible and arguing against purely genomic explanations. Although cosegregation is statistically significant, because our polymorphism data set is limited (compared to whole-genome comparisons of divergence) there is more uncertainty about these results, and it is worth noting...
haplotype for polymorphisms a distance apart, \( p_m \) is the frequency of the derived allele at site \( i \). \( \langle D_y(y) \rangle \) is averaged over all pairs of polymorphisms \( y \) apart, \( \langle D_y(y) \rangle > 0 \) indicates an overrepresentation of the doubly derived haplotype, which we also refer to as preferential cosegregation of the derived alleles. We test the significance of cosegregation by bootstrapping, which yields \( p = 2 \times 10^{-4} \) that as great or greater cosegregation of nearby derived alleles (as measured by \( \langle D_y(y) \rangle \) for \( y \in \{1, \ldots, 3\} \)) would be observed by chance.

Figure 6. Polarized linkage disequilibrium. Doubly derived haplotypes are overrepresented among nearby pairs of non-synonymous polymorphisms (PN-PN) in our population sample, but not when one (PN-PS), or both (PS-PS), of the polymorphisms are synonymous. This is quantified by the average polarized linkage disequilibrium \( \langle D_y(y) \rangle = \langle p_{m,y} - p_m p_y \rangle \), where \( p_{m,y} \) is the frequency of the doubly derived haplotype for polymorphisms \( y \) apart and \( p_y \) is the frequency of the derived allele at site \( i \). \( \langle D_y(y) \rangle \) is averaged over all pairs of polymorphisms \( y \) apart, \( \langle D_y(y) \rangle > 0 \) indicates an overrepresentation of the doubly derived haplotype, which we also refer to as preferential cosegregation of the derived alleles. We test the significance of cosegregation by bootstrapping, which yields \( p = 2 \times 10^{-4} \) that as great or greater cosegregation of nearby derived alleles (as measured by \( \langle D_y(y) \rangle \) for \( y \in \{1, \ldots, 3\} \)) would be observed by chance.

doi:10.1371/journal.pgen.1001315.g006

Figure 6. Polarized linkage disequilibrium. Doubly derived haplotypes are overrepresented among nearby pairs of non-synonymous polymorphisms (PN-PN) in our population sample, but not when one (PN-PS), or both (PS-PS), of the polymorphisms are synonymous. This is quantified by the average polarized linkage disequilibrium \( \langle D_y(y) \rangle = \langle p_{m,y} - p_m p_y \rangle \), where \( p_{m,y} \) is the frequency of the doubly derived haplotype for polymorphisms \( y \) apart and \( p_y \) is the frequency of the derived allele at site \( i \). \( \langle D_y(y) \rangle \) is averaged over all pairs of polymorphisms \( y \) apart, \( \langle D_y(y) \rangle > 0 \) indicates an overrepresentation of the doubly derived haplotype, which we also refer to as preferential cosegregation of the derived alleles. We test the significance of cosegregation by bootstrapping, which yields \( p = 2 \times 10^{-4} \) that as great or greater cosegregation of nearby derived alleles (as measured by \( \langle D_y(y) \rangle \) for \( y \in \{1, \ldots, 3\} \)) would be observed by chance.

doi:10.1371/journal.pgen.1001315.g006

Discussion

We have shown that the presence of an amino acid substitution substantially increases the probability of there being additional amino acid substitutions nearby in the protein sequence, with the strength of this effect decaying exponentially along the sequence with a characteristic length scale of \( \approx 10 \) codons. This ‘clustering’ phenomenon is not observed for synonymous substitutions and is insensitive to the presence of intervening intronic sequence, strongly suggesting selection on proteins as the root cause. Both divergence between Drosophilids and polymorphisms within a population sample of \( D. melanogaster \) exhibit this effect. Clustering has a substantial lineage-specific component and nearby substitutions in the same lineage tend to conserve local charge, suggesting compensatory evolution plays a role.

While the results presented here are derived from Drosophila data, we expect that clustering obtains more generally. A recent study found that mutations identified as compensatory clustered near their associated deleterious mutations in eukaryotes, prokaryotes and viruses [37]. Similarly, nucleotide substitutions cluster within codons more often than expected in mammals and HIV, suggesting that two successive mutations are required for the incorporation of some fraction of amino acid substitutions [38,39].

Origin of clustering

There are a number of selective mechanisms that could cause amino acid substitutions to cluster, and the clustering we observe most likely has multiple causes. We will now try to reconcile the various observations made above with the different mechanisms that have the potential to cause clustering, and estimate their respective contributions. Potential selective mechanisms of clustering can be grouped into two classes: (A) Heterogeneity in the strength of purifying selection acting within an ORF leads to variation in the density of substitutions and polymorphisms, resulting in clustering. (B) Novel protein variants are selected for and this adaptation leads to clusters of substitutions. The latter class of mechanisms comes in several flavors: (i) A localized adaptive burst in which several nearby substitutions independently sweep to fixation. This might be a consequence of changes in selective pressure on a protein domain that requires multiple adaptive substitutions to reach the new optimum [40]. (ii) A complex adaptation, in which several dependent substitutions are required to achieve the selected effect. This case includes scenarios of compensatory mutations, i.e. a second mutation is necessary to compensate deleterious side effects of the first [41], and evolutionary contingency, i.e. the first mutation is necessary for the second mutation to be beneficial [42]. (iii) Hitchhiking, the fixation of otherwise deleterious substitutions as a result of a selective sweep at a linked site [43,44].

Purifying versus positive selection

Purifying selection prunes mutations that are detrimental, perhaps because they interfere with protein structure or stability. Given that protein structure is strongly conserved across different domains of life, it is reasonable to assume that purifying selection operates in a similar fashion on homologous regions of proteins in different branches of the Drosophila phylogeny. Adaptive evolution, however, depends on the ecological niche of the species and can depend strongly on previous substitutions in that species. Adaptive evolution is therefore expected to be lineage-specific, at least moreso than purifying selection.
We observed that clustering exists between pairs of amino acid substitutions in different lineages as well as in the same lineage, the latter being consistently greater (Figure 4). Clustering across lineages implies that a substitution found in one lineage is predictive of the local substitution rate independent of lineage, which we understand as a lineage-non-specific local increase in the substitution rate. This is most consistent with a class (A) mechanism such as locally relaxed purifying selection, e.g. in an unstructured loop of a protein.

The excess clustering within lineages must be caused by a lineage-specific mechanism such as the class (B) mechanisms described above. Purifying selection can of course also vary in a lineage-specific way. If mildly-deleterious substitutions were highly clustered, and a reduced effective population size rendered them effectively neutral, this could result in excess clustering in the lower population size lineage. However, this scenario is inconsistent with the fact that we observe excess clustering within all lineages, and that it is quantitatively similar between lineage pairs diverging from a common ancestor. Locus-specific variation in purifying selection is also possible, but in most cases will affect an entire gene (e.g. via duplication or transformation into a pseudo-gene) and therefore would not lead to clustering on short length scales. Given that excess lineage-specific clustering is a substantial fraction of the total clustering in every lineage, it does not seem likely that lineage-specific variation in the strength of purifying selection can account for it.

Adaptive mechanisms for clustered substitutions

We start by addressing the potential contribution of hitchhiking to clustering. A selective sweep of a strongly beneficial substitution fixes a linked haplotype, converting a local snapshot of polymorphisms present in the population into substitutions. This hitchhiking process does not affect the fixation probability of neutral (and perhaps synonymous) mutations [45], but is expected to increase the fixation probability of nearby deleterious non-synonymous substitutions. However, several observations argue against hitchhiking as the main contributor to clustering. First, hitchhiking predicts that the length scale of clustering is given by the typical size of hitchhiked region [46]. This implies clustering dependent on separation along the DNA sequence rather than along the protein backbone, contrary to our observations (Figure 2B). Second, there is no correlation between clustering and the average recombination rate of a coding sequence, which would affect the size of hitchhiked regions (Figure S3). Finally, we can calculate a rough upper bound for the contribution of hitchhiking to lineage-specific clustering. Given a per-site heterozygosity \( \pi \), the expected population frequency of derived mutations per site is \( \pi/2 \). Non-synonymous \( \pi \) in \( D. melanogaster \) is \( \sim 0.0018 \) per site [47–49] and thus \( \sim 0.004 \) per 4-fold codon (and slightly higher for 2-folds). Given this, the probability of finding a derived amino acid substitution within \( L = 20 \) codons of a focal site is \( \sim L \star (\pi/2) \sim 20 \star (0.002) = 0.04 \). This serves as a very generous upper bound on the contribution of hitchhiking to \( \Delta \Omega \), since only if the focal site is always adaptive and the observed variation always deleterious will this value be approached. This estimate suggests that the contribution of hitchhiking to lineage-specific clustering is minor, since this upper bound is less than the range over which we observe lineage-specific excess, from 0.04 to 0.15 depending on lineage (Figure 4B).

The two remaining adaptive scenarios, adaptive bursts and complex adaptations, are difficult to distinguish in part because the boundary between them is not sharply delineated. Certainly, different substitutions within 10 codons in the same protein are never going to be completely independent. The question rather is whether one of the mutations ‘substantially’ affected the probability of the other. Do localized adaptive bursts, loosely defined as \( \geq 2 \) substitutions within \( \sim 10 \) codons which all independently improve fitness, dominate our clustering signal? Or are the interactions (epistasis) between nearby substitutions mainly responsible? We cannot fully exclude either scenario, but there is evidence that local interactions play at least a significant role. Mutations of independent beneficial effect would not be expected to compensate each others effect on total charge. This requires epistasis between the substitutions, and implies that complex adaptations are responsible for at least 5–10% of lineage-specific excess. Secondly, independent beneficial mutations are expected to either fix sequentially or, if they do occur simultaneously, to generally segregate in repulsion [50]. This is inconsistent with the preferential cosegregation we observe between nearby derived alleles (Figure 6). Furthermore, charge compensation is only one of many relevant interactions, albeit the one we most readily ascertained from the primary sequence data. So the contribution of charge compensation is only a lower limit for the influence of complex adaptation on the substitution process.

While the possibility of interactions between amino acid substitutions has never been seriously questioned (and has recently been demonstrated in a number of concrete examples [42,51]), the general importance of epistasis and compensation in evolution has been, and continues to be, controversial. We find evidence that a non-negligible fraction of substitutions are involved in patterns of adaptation suggestive of epistasis. If lineage-specific clustering is mostly due to epistasis, a scenario consistent with our results, we can use the lineage-specific excess to estimate the number of substitutions which owe their fixation to interactions with other substitutions. For example, the lineage-specific excess in the D. yakuba lineage is \( \Delta \Omega(D_{\text{Dyak}}) = 0.07 \). If we attribute the entirety of this to epistasis we would conclude that \( \sim 7\% \) of the substitutions on this lineage were contingent on another substitution. This estimate is clearly generous in the sense that we have not completely excluded the contribution of other processes, but it is also conservative in the sense that it only includes the effect of elevated local epistasis and excludes the contribution of long-range interactions.

To account for interactions between amino acids distant in the protein sequence but nevertheless in close vicinity in the folded protein, one would need to incorporate protein structure explicitly. However, the probability for any random pair of residues to be involved in such interaction decays rapidly with their separation along the protein backbone, likely to an asymptotic value. Hence, in our analysis we expect correlations between distant pairs to be lost in the background, with only the enriched short range interactions observable as excess clustering of substitutions. The presence of this local enrichment is the enabling factor behind our approach. In agreement with this interpretation, the inferred length scale of clustering of 10 codons is consistent with the size of secondary structure elements in proteins (e.g. 3 turns of an \( z \) helix). While this manuscript was prepared for publication, another group also found clustering of positively selected amino acid substitutions [17]. Via a different approach, the authors show that the rate of evolution depends on elements of secondary structure and that nearby positively selected sites tend to cluster.

Finally, while we have focused on the mode of evolution responsible for lineage-specific excess, the clear clustering which occurs across lineages is notable in its own right. We attribute this clustering to spatially heterogeneous purifying selection. The clustering length scale is extremely consistent across all the species comparisons we considered and the polymorphism data (Figure 2). This suggests that models of protein evolution might be improved by incorporating correlation between the rate of amino acid
evolution along the sequence (e.g. site-specific Ω in PAML [32]).
This is particularly true if the length scale we observe here can be
to be consistent across phyla, demonstrating it as a generic
property of proteins themselves.

**Methods**

We assign substitutions in coding sequence (CDS) on a codon-
by-codon basis to pairwise alignments of the reference genome of
*D. melanogaster* with the reference genomes of 6 other Drosophilids:
*D. sechellia*, *D. simulans*, *D. yakuba*, *D. erecta*, *D. ananassae* and *D.
pseudoobscura*. We use FlyBase release 5.26 gene models to identify
the location of coding sequence in the *D. melanogaster* genome.

Coding sequence substitutions are assigned only in the absence of
gaps and ambiguous nucleotide. If the aligned codons encode
different amino acids a non-synonymous substitution (DN) is
assigned, if the same amino acid, a synonymous substitution (DS).

Substitutions are assigned in the context of an alignment between
two lineages, which we can make explicit by writing DN(xβ)
where xβ is the pairwise alignment between lineages x and β. We
omit the alignment for notational convenience, the alignment under consideration will be clear by context, but it is worth remembering that the objects we define later depend implicitly on
an alignment when they involve substitutions.

Substitutions are polarized into the lineage in which they arose
by comparison to the closest available Drosophilid that is more
distant from *D. melanogaster* than the one being aligned. Specifically,*
*D. yakuba* is used as the outgroup for the *D. simulans* and *D. sechellia*
comparisons, *D. ananassae* for *D. yakuba* and *D. erecta*, *D.
pseudoobscura* for *D. ananassae* and *D. willistoni* for *D. pseudoobscura*.

Substitutions are assigned to a lineage γ if the assignment is
unambiguous using standard parsimony criteria. A DN polarized
into alignment γ is denoted DNγ. Not all substitutions can be
polarized, zAB represents the fraction of substitutions between
the lineages x and β which are polarized. The species comparisons
between *D. melanogaster* and either *D. yakuba* or *D. erecta* have
the best properties for the analysis here: most coding sequence
alignments pass quality checks and the number of substitutions,
both polarizable and total, is high, as seen in Figure S1 and
observed previously [53]. When we present results from just one
species comparison it will be the *D. melanogaster* - *D. yakuba*
comparison for this reason.

Having assigned and polarized substitutions, we study clustering
between substitutions typed by synonymy, e.g. non-synonymous
(DN) and synonymous (DS) divergent sites, by estimating the
probability of finding a substitution (of some particular type) y
codons away from a focal substitution. This is formalized as a
conditional probability distribution (cPDF), which we denote
codons away from a focal substitution. This is formalized as a
probability of finding a substitution (of some particular type)
(DN) and synonymous (DS) divergent sites, by estimating the
expected number of substitutions within 20 codons downstream of
a focal f substitution and the expected number in a 20 codon
segment that is distant from the focal substitution. The choice of 20
as the upper limit of the sum simply reflects the
observation that significant clustering does not extend past this
point. Additionally, this is a one-sided sum, ensuring that each pair
is counted only once. We also define the lineage-specific excess
clustering count for lineage x, ΔΩflows(x), in order to quantify the
stronger clustering within a lineage. Lineage-specific excess is
found by summing over the difference between the normalized
same-lineage cPDF and the normalized cross-lineage cPDF and
then ‘unnormalizing’,

\[
\Delta \Omega_{f}^{(y)}(x) = \left( \sum_{y=1}^{20} \left( C_{y}^{(f)}(y) - A_{f}^{(y)} \right) \right) \times A_{f}^{y} \times z_{AB}^{-1}
\]  

The factor of \( z_{AB}^{-1} \), defined above, corrects \( A_{f}^{y} \) for the fraction of
substitutions that cannot be unambiguously polarized. Multi-
plying by \( z_{AB}^{-1} \) roughly accounts for this by assuming that the
polarized substitutions are representative of the unpolarized ones.
When Ω or ΔΩ are written without indices they should be assumed
to refer to DN clustering, i.e. \( \Omega_{DN} \). Note that Ω and ΔΩ depend
on the pairwise alignment zβ being considered via their
dependence on the assignment of substitutions, as described at
the beginning of the Methods.

Our definition of cPDFs implicitly involved the determination of
the set of CDSs to be summed over. This set varies with the species
comparison so we denote it \( \{ v \}_{zβ} \). For the results presented here
\( \{ v \}_{zβ} \) was set as the set of all CDSs for which the pairwise alignment
between *D. melanogaster* and sister Drosophilid met several
standards of quality: a CDS included in \( \{ v \}_{zβ} \) was required to
have less than 20% gapping in its pairwise alignment and less than
20% amino acid substitution, the alignment could not contain out-
of-frame gaps (gaps with size that is not a multiple of three), and
the spliced transcript to which the CDS belongs could contain no
extraneous stop codons. Furthermore, we often restrict \( \{ v \}_{zβ} \) to a
specific subset in order to investigate the dependence of the clustering signal on various quantities, e.g. Figure 3B shows the cPDFs calculated using subsets of all CDNs ranked by $D_n$.

Polymorphisms were identified in a Zambian population sample of male D. melanogaster. We re-sequenced 130 kb of coding sequence from 182 genes in the highly recombinating region of the X-chromosome (cytological positions 3C3 to 18F4) using standard methods reported previously [34]. Samples sizes ranged from 14 to 26 with a mean of 22. A subset of these sequences (12 alleles for each of 137 loci), were previously reported [34]. All new sequences have been submitted to GenBank, accession numbers are available in Table S3. Polymorphisms are assigned if more than one codon exists in the population sample at that site. Singletons are excluded from the analysis. A non-synonymous polymorphism is assigned if this set of codons encodes more than one amino acid, and a synonymous polymorphism if the number of codons exceeds the number of amino acids encoded. PN and PS assignment is not exclusive. Polarization into mutant/ancestral alleles is inferred by comparison to D. simulans (or D. sechellia when D. simulans is unavailable) at that site (i.e. standard parsimony criteria). cPDFs are constructed analogously to those involving substitutions.

All line plots presented are smoothed from the underlying data. We used a moving window averaging for smoothing, always with window size 5. The contribution from each data point to the smoothed average was weighted by the number of ‘trials’ from which the value was estimated.

Assessment of significance

We assess two ‘types’ of significance here, sampling significance and bootstrapping significance. The assessment of sampling significance is understood by recalling how cPDFs are estimated. $C_f (y)$ is the mean of a set of trials which can have outcome either 0 or 1 (Bernoulli random variables). $Q_f$ is the same, it is just an average over a cPDF for $y \in [1 - 20]$. Trials consist of selecting a focal substitution of type $j$, looking away on the sequence, and recording the presence (1) or absence (0) of a substitution of type $j$'. So, assessing the significance of values of $C_f (y)$ or $Q$ is equivalent to assessing the significance of sums of Bernoulli random variables, for which we used chi-square tests.

Bootstrapping significance is also a measure of sampling significance, with the difference being that the effect of resampling is evaluated at the level of the largest unit in our analysis, the coding sequence. The probability distribution of a value of interest is constructed by resampling with replacement from the full set of coding sequences a ‘bootstrapped’ set of equal size, estimating the value of interest on that bootstrapped set, and repeating. Bootstrapping p-values are then determined from this estimate of the probability distribution. If the estimated distribution can be approximated as a gaussian, as is always the case here, the gaussian approximation is used to assign the p-value. A modification of this bootstrapping scheme was used for polymorphism cosegregation, and described there.

Supporting Information

Figure S1 Numbers of substitutions included in our analysis by species comparison. The number of substitutions included in our analysis varies with the species comparison considered. Increased species divergence increases the fraction of substituted sites, while sequence/alignment quality affects the proportion of coding sequence alignments which meet our quality thresholds (Methods). Polymorphism substitutions are those which can be unambiguously assigned to one lineage or the other by parsimony with the closest outgroup (Methods). The total number of codons in qualified coding sequences (divided by 10) is shown for reference. The species comparison of D. melanogaster to D. yakuba or D. erecta have the best statistical properties for our analysis, most coding sequences have qualifying alignments and the total number of substitutions is high.

Found at: doi:10.1371/journal.pgen.1001315.s001 (0.01 MB PDF)

Figure S2 Dependence of clustering on chromosome. A) The cPDFs $C_{DN} (y)$ (solid lines) and $C_{DS} (y)$ (dashed lines) are estimated from chromosome-specific sets of coding sequences, for the D. melanogaster to D. yakuba species comparison. Little variation is found, even between the sex-linked X chromosome and the autosomes. B) Chromosome-specific $\Omega_{DN}$, $\Omega_{DS}$ and $\Omega_{DS}$ are plotted versus the chromosome over which they are estimated. Clustering is consistent across all chromosomes.

Found at: doi:10.1371/journal.pgen.1001315.s002 (0.03 MB PDF)

Figure S3 Dependence of clustering on recombination rate (cM/Mb). A) The cPDFs $C_{DN} (y)$ (solid lines) and $C_{DS} (y)$ (dashed lines) are estimated from each decile of the full set of X-chromosome coding sequences ranked by average recombination rate, for the D. melanogaster to D. yakuba species comparison. No systematic relationship between recombination rate and clustering is observed. B) $\Omega_{DN}$, $\Omega_{DS}$ and $\Omega_{DS}$ are plotted versus the average recombination of each ranked decile. The lack of a relationship between recombination rate and clustering is confirmed. The sex-averaged recombination rate was estimated using 149 point estimates of the sex-averaged recombination rate (cM/Mb) across the X chromosome in D. melanogaster (Begun et al. 2007), the local recombination rate for each coding sequence was estimated by linear interpolation. The recombination rates at the telomere and centromere were assumed to be zero. Only the X chromosome was used because recombination rate is more accurately described on the X.

Found at: doi:10.1371/journal.pgen.1001315.s003 (0.05 MB PDF)

Figure S4 The dependence of clustering on gapping in the alignment. The cPDFs $C_{DN} (y)$, $C_{DS} (y)$ and $C_{DS} (y)$ are estimated from the sets of coding sequences with and without gaps in their alignment for the D. melanogaster to D. yakuba species comparison. Alignments with gaps are expected to be of lower quality, potentially introducing an artefactual clustering signal. While the gapped sequences have slightly higher clustering, the difference is quantitatively slight and there is no qualitative difference. This suggests that misalignment is not the root cause of clustering.

Found at: doi:10.1371/journal.pgen.1001315.s004 (0.02 MB PDF)

Figure S5 Dependence of clustering on GC content. A) The cPDFs $C_{DN} (y)$ (solid lines) and $C_{DS} (y)$ (dashed lines) are estimated from each decile of the full set of coding sequences ranked by their GC content for the D. melanogaster to D. yakuba species comparison. Increased GC content correlates with greater clustering, as is seen by the higher peak of $\Omega_{DN}$, $\Omega_{DS}$ and $\Omega_{DS}$ from their asymptotic value is shown for the D. melanogaster to D. yakuba species comparison.

Found at: doi:10.1371/journal.pgen.1001315.s005 (0.05 MB PDF)

Figure S6 Unnormalized cPDFs between substitutions typed by synonymy. The deviation of the unnormalized cPDFs $C_{DN} (y)$, $C_{DS} (y)$ and $C_{DS} (y)$ from their asymptotic value is shown for the species comparison of D. melanogaster to D. yakuba. While there is a small amount of synonymous clustering at very short scales, as evidenced by the small peak in $C_{DN} (y)$ and $C_{DS} (y)$ near $y = 0$, it is
clear that this effect is an order of magnitude less than the non-synonymous clustering \( C^{DN}_{DN}(y) \). This suggests that the mechanism primarily responsible for non-synonymous clustering does not apply to synonymous substitutions.

**Figure S7** 1,000 bootstrapped estimates of unnormalized cPDFs typed by synonymity. 1,000 Bootstrapped estimates of the unnormalized cPDFs \( C^{DN}_{DN}(y) \), \( C^{DS}_{DS}(y) \) and \( C^{DS}_{DN}(y) \) are plotted for the species comparison of \( D. melanogaster \) to \( D. yakuba \). The consistency of the cPDFs suggests that the conclusions we draw from this data, in particular that synonymous clustering is negligible relative to non-synonymous clustering, are not artefacts of a few anomalous genes, but instead reflect a generic characteristic of gene evolution.

**Figure S8** 1,000 bootstrapped estimates of non-synonymous and synonymous clustering counts. The clustering counts \( \Omega^{DN}_{DN}, \Omega^{DS}_{DN} \) and \( \Omega^{DS}_{DS} \) are estimated from 1,000 bootstrapped replicates of the \( D. melanogaster \) to \( D. yakuba \) species comparison. Non-synonymous clustering \( \Omega^{DN}_{DN} \) is an order of magnitude larger than the synonymous clustering counts \( \Omega^{DS}_{DN} \) and \( \Omega^{DS}_{DS} \), both of which have bootstrapping distributions overlapping zero. We can extract a bootstrapping p-value from these distributions of \( p < e^{-100} \) for the hypothesis that \( \Omega^{DN}_{DN} \leq 2 \Omega^{DS}_{DN} \) (Methods), which roughly corresponds to the hypothesis that the mechanism responsible for clustering is blind to codon structure (non-selective). The factor of two reflects the roughly double target size for non-synonymous errors (first two positions versus third position).

**Figure S9** The dependence of clustering on alignment methodology. The cPDFs \( C^{DN}_{DN}(y), C^{DS}_{DS}(y) \) and \( C^{DS}_{DN}(y) \) are estimated from the alignments used in this paper (blatz genome alignments) and from coding sequence alignments of selected orthologs made publicly available as part of the twelve species analysis at ftp://ftp.drosophila.net/geneomes/12_species_analysis/clark_eisen/alignments/. In both cases the same underlying sequences are being used, but the selection of orthologs and alignment methodology are different. The concordance between the clustering observed in both cases indicates that clustering is not an artefact of some detail of the alignment methodology.

**Figure S10** Bootstrap distributions of \( \Delta \Omega^{DN}_{DS}(x)/D_{x}(z) \) by lineage \( x \), \( \Delta \Omega^{DN}_{DS}(x)/D_{x}(z) \) is the lineage-specific excess of synonymous substitutions near non-synonymous substitutions, normalized by the overall level of synonymous divergence in the lineage \( x \) - \( D_{x}(z) \). Consideration of this quantity serves as a particularly effective synonymous control for the contribution of non-selective processes to \( \Delta \Omega^{DN}_{DS} \). The bootstrap estimates of the probability distribution of \( \Delta \Omega^{DN}_{DS}(x)/D_{x}(z) \) (Methods) are plotted here for every lineage included in this study. Every distribution overlaps zero, suggesting little to no contribution to clustering from non-selective processes, except for the Dsec and Dsm lineages. Both of these lineages have significant lineage-specific synonymous clustering with the effect in Dsm being particularly strong. These results suggest that some non-selective process (such as sequencing error) could be contributing to the lineage-specific clustering in these lineages, and \( \Delta \Omega^{DN}_{DS} \) in particular.

**Table S1** Magnitude and significance of lineage-specific clustering \( \Delta \Omega(z) \). The magnitude of \( \Delta \Omega(z) \) is recorded for both lineages of each species comparison considered. All lineages have positive \( \Delta \Omega(z) \), which indicates that clustering is stronger within that lineage than it is between that lineage and the lineage to which it is being compared. The significance of this is quantified by calculating the p-value for the hypothesis that \( \Delta \Omega(z) = 0 \). Both a sampling p-value, calculated using a chi-square test, and a bootstrapping p-value, calculated using the bootstrap estimate of the probability distribution of \( \Delta \Omega(z) \), are determined (Methods). Every lineage has significant excess lineage-specific clustering, using either measure of significance.

**Table S2** Correlations between charge-altering substitutions as a function of sequence separation. The hypothesis that the fraction of substitutions causing correlated changes in charge is elevated when the substitutions are within 10 codons of one other is tested. Pairs of substitutions are typed by whether the substitutions occurred on the \( \{ \)same or \( \} \)ifferent lineages. Conditioned on the focal divergence altering charge, the fraction of substitutions a distance \( y \) away which are \( \{ c \} ompensatory and \( r \) einforcing are considered, compensation being charge alterations in opposite directions, and reinforcement when in the same direction. The chi-square p-values are listed, with subscripts indicating the lineage condition and charge relationship being tested, e.g. \( p_{SC} \) is the p-value for the hypothesis that same-lineage substitutions within 10 codons of each other do not have an increased probability to cause compensating changes in charge compared to same-lineage substitutions distant from one another. We find that charge compensation is significantly more frequent among nearby substitutions in the same lineage, but not when the substitutions arose on different lineages. Interestingly, nearby substitutions on different lineages do have a consistently significant increased frequency to cause positively correlated changes in charge, perhaps indicating convergent evolution. The ‘fraction compensated’ is the average net charge compensation caused by same-lineage substitutions within 10 codons of a focal charge-altering substitution. The contribution of local charge compensation to lineage specific excess clustering \( \Delta \Omega(z) \) is also estimated for each lineage. Local charge compensation consistently accounts for between 5% and 10% of \( \Delta \Omega(z) \).

**Table S3** Accessions numbers for sequences from our population sample. We re-sequenced Zimbabwean population sample of male \( D. melanogaster \) at loci in 182 genes in the highly recombining region of the X-chromosome (cytological positions 3C3 to 18F4) using standard methods reported previously [54]. The GenBank accession numbers of these sequences are shown here.

**Acknowledgments** We thank Guy Sella for sharing his observations on clustering of amino acid substitutions in the Drosophila data, Dan Davison for comments on the manuscript, Karen Wong for technical assistance. We acknowledge stimulating discussions with Martin Kreitman and other participants of the “Population Genetics and Genomics” program at the KTFP, where this work was initiated.

**Author Contributions** Conceived and designed the experiments: BC BIS. Performed the experiments: DB PA. Analyzed the data: BRAN. Contributed reagents/materials/analysis tools: BC DB PA. Wrote the paper: BC RAN PA BIS.
Amino Acid Changes Cluster in Protein Sequence

References