# A putative bacterial ecocline in *Klebsiella pneumoniae*

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## Abstract

The genetic structure of bacterial species is most often interpreted in terms of clonal descent but can also reflect processes including natural selection, and hence give functional and ecological insight. *Klebsiella pneumoniae* (KP) has high recombination rates and disperses effectively around the world which should result in the species having a well-mixed gene pool. Nevertheless, phylogenies based on diverse KP strains contain a "backbone". This structure reflects a component of variation captured by the first component, PC1, in Principal Components Analysis (PCA) which explains 16.8% of total variation. We propose that the component is generated by diversifying selection on a quantitative genetic trait. We simulated a model in which trait is influenced by many genes, and strains with the most extreme trait values have a small advantage. For high recombination rates or weak selection, the simulated gene pool is approximately randomly mixed, with low variation in trait values. For intermediate rates, trait values become continuously distributed over a wider range. We call the gradient in gene frequencies of the genes associated with the trait a bacterial ecocline. For low recombination rates or stronger selection, trait values the species splits into two separate gene pools. After fitting selection and recombination parameters, we were able recapitulate the overall genetic structure of KP, while the neutral models we investigated could not. As well as providing an explanation for the phylogenetic backbone, our results provide insight into how species such as KP can speciate, via stronger selection on a trait or a reduction in gene flow. Our hypothesis begs two questions about the putative KP bacterial ecocline, namely what the trait is underlying it and why is it under diversifying selection. The genes that are most strongly associated with PC1 provide some hints with number 1 being the fimbria locus *Kpa*. Identification of the trait, if it exists, should facilitate insight into selection on quantitative genetic traits in natural bacterial populations, which have largely been unstudied in microbiology, except in the atypical context of antibiotic resistance.

## Introduction

When looking at the phylogenetic trees of bacteria, what information can we extract[1]? The shape of the trees reflects key demographic factors, such as clonal relationships or geographic structure shaped by barriers to dispersal and genetic exchange[2]. Deep splits within trees and an absence of intermediate strains reflect long-standing divergence between groups, that can arise due to niche specialization and/or low levels of genetic exchange between groups[3,4]. However, many species show a pattern of differentiation that is more continuous in character, which can be harder to explain.

*Klebsiella pneumoniae* (KP) is a major human pathogen capable of causing severe infections in immunocompromised individuals[5], but is found in a wide variety of environments[6], including soil[7], wastewater[8], and even the ocean[9]. Like many other bacterial species, KP has an enormous population size, exhibits high transmission efficiency and has high recombination rates between strains[10]. As a result, its global genetic diversity can be captured from samples collected in a single region, whether from Europe[11], Africa[12], Asia[13], or elsewhere. Despite these features, the phylogenetic tree shows deep structure, with strains radiating from multiple points along a "backbone". Until now, to our knowledge, no explanations have been proposed for the shape of the tree.

We propose that the phylogenetic tree shape of KP reflects diversifying selection[14] acting on a quantitative trait[15]. We simulate a population of bacteria with a single phenotypic trait and show that if strains exhibiting extreme trait values have a fitness advantage, we can recapitulate patterns of diversity found in KP. We call the gradient in gene frequency values that arises a bacterial ecocline. Quantitative traits are central to our understanding of genetic diversity in animals and plants but have hitherto been largely overlooked for bacteria. If we can identify the trait or traits under selection in KP, it should provide considerable insight into the genetic architecture of bacterial quantitative traits as well as the fitness trade-offs[16] that the species experiences.

## Results

### The phylogenetic backbone reflects a component of variation

Genetic relationships amongst bacteria are most often represented using a phylogenetic tree[1,17]. Trees based on core genome SNPs from global collections *Klebsiella pneumoniae* (KP) isolates are "star-like" (Figure 1a & Supplementary Figure 1, non-redundant dataset, with clonally related strains removed; Supplementary Figure 2, entire dataset), with many branches of approximately equal length. The shape of the tree reflects extensive

recombination, with the length of the branches reflecting the overall level of diversity in the gene pool[10,18]. However, the overall shape of the KP tree is oval rather than circular as for example seen in *Vibrio parahaemolyticus*[19,20] and KP lineages, instead of radiating from a single point, are connected by a "backbone". This backbone is present in any phylogeny drawn based on a diverse sample of KP genomes but it may be less visually evident than in Figure 1A, depending on the strains sampled, the rooting of the tree, and the phylogenetic and plotting algorithms.

The backbone of the KP tree is a line, from which multiple branches radiate, at different points along its length. Informally, the backbone has a top and a bottom, which are marked in Figure 1a. The strains that branch at the top could be given maximum backbone scores and those at the bottom minimum ones, with intermediate strains given values according to how far along the line they branch. We have not defined scores in this way, because there is a more systematic way of describing this variation, namely Principal Components Analysis (PCA)[21,22].

PCA identifies axes of variation that together explain the overall diversity within the species, with the first component, PC1, explaining the largest fraction of variation. When PCA was applied to the non-redundant global KP core genome SNP dataset used to construct the tree in Figure 1A, the first component (PC1) explained 16.7 % of the variation, with subsequent components each explaining approximately 8% or less (Figure 1b). PC1 captured the genetic variation underlying the phylogenetic backbone of the species, since strains with extreme PC1 values are found at either end of the backbone, while strains branching from the middle have intermediate values (Figure 1a). Strain loadings for PC1 are approximately continuous, albeit with a paucity of intermediate values (Figure 1b).

PCA is sensitive to all signals of relatedness, including clonal structure and can be prone to overfitting, especially where there are orders of magnitude more genetic markers than strains[23]. However, we found that PC1 was reproducible in different analyses of non-redundant datasets (Supplementary Figure 3). Specifically, PC1 values obtained when performing analysis on one half of the chromosome were correlated with those of PC1 calculated using the other half, implying that the signal is not due to specific genetic loci (Supplementary Figure 3 e & f). PC1 strain loadings were also consistent if components were defined by strains on the right-hand side of the oval or those on the left, implying that they are not generated by clonal structure (Supplementary Figure 3 a-d). They were also consistent when calculated using SNPs with extreme PC1 loadings or loadings that are closer to 0 (Supplementary Figure 3 g-j), implying that the signal affects genetic variation genome-wide.

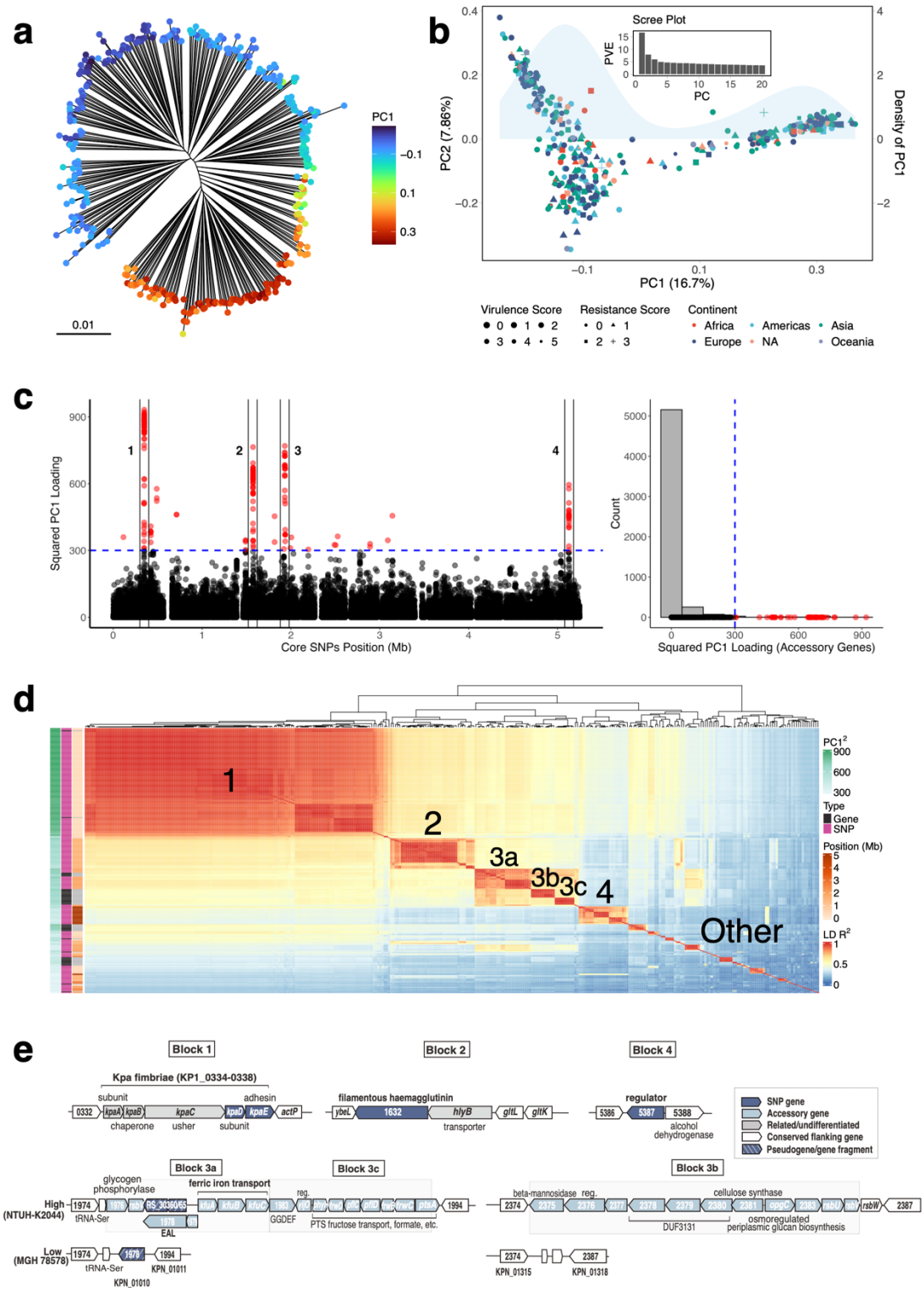


**Figure 1.** Population structure and genetic variation in a non-redundant KP dataset.

(a) Maximum-likelihood phylogenetic tree of non-redundant KP based on core SNPs, with a color

gradient showing PC1 variation. (b) PCA of non-redundant KP based on the pruned SNPs. Colors indicate geographic origin, while marker size and shape represent resistance and virulence scores, respectively, determined by Kleborate[24]. Sky blue shadow represents the density of PC1 values. The inset scree plot shows the variance explained (PVE) by the first 20 principal components. (c) Manhattan plot of squared core SNP (left) and accessory genome (right) loadings for PC1. Variants with values greater than 300 are defined as high loading variants and are marked in red. (d) Heatmap of pairwise linkage disequilibrium (LD) $R^2$ values for high loading variants, with the first four blocks (including subblocks) labelled. (e) Functional annotation of blocks 1-4.

To benchmark these results, we applied the same methods to data of a simulated neutrally evolving bacterial population, which will be discussed in more detail below. For the simulated data, PC1 does not explain as much of the variance and the reproducibility of the cline in all three tests for the KP data contrasts with the irreproducibility of clines seen in a simulated neutrally evolving dataset, where no correlation is seen when PCA is applied separately to the strains on the right-hand side and left-hand side of the phylogenetic tree (Supplementary Figure 4). Thus, according to these criteria, we can falsify the simplest neutral models based on these results.

*Vibrio parahaemolyticus* (VP) also represents a useful benchmark for half-matching tests as it has well characterized geographical population structure and ecospecies structure[19,20,25]. The population structure arises because the species lives in coastal waters, and there have been barriers to movement of bacteria between oceans, albeit disrupted by recent human activity. VP also has an ecospecies EG1a which is found in different geographical populations where it has fixed differences with non-EG1a strains in at least 27 core genes, while undergoing high rates of recombination in the rest of the genome[20].

When half matching tests were applied to a global non-redundant dataset of VP, all three half-matching tests gave reproducible results that reflected the population structure in the dataset (Supplementary Figure 5). However, when analysis was restricted to the Asian population (VppAsia), ecospecies structure generated weaker correlations in two of the three tests but there was no correlation between low loading and high loading loci, consistent with a single randomly mixing gene pool for most the genome[20,25] (Supplementary Figure 6). Thus, the reproducibility of PC1 in all three tests in KP shows that the structure is genome-wide and in this respect is more like geographic population structure and less similar to ecospecies structure, which is only present in a fraction of the genome[25].

**Loci strongly associated with PC1 are localized in the genome**

In addition to strain loadings (Figure 1b), PCA generates SNP loadings, indicating which

genomic variants are most strongly associated with the component. SNP loadings for PC1 showed four clear peaks (Figure 1c, left). Each peak contained many SNPs within less than 10kb (Supplementary Fig. 7a). We also calculated loadings for accessory gene presence-absence, based on a Panaroo-generated[26] pangenome of the non-redundant strains, and found many had high loadings (Figure 1C, right).

To investigate the relationships between the loci most strongly correlated with PC1, we chose a cutoff of 300 for squared PC1 loadings and calculated pairwise LD values for all SNPs and genes above the cutoff. Hierarchical clustering of LD values organized the extreme loading variants into 44 blocks (Figure 1d). Four of these blocks include the SNPs with the most extreme loadings within the four peaks seen in Figure 1C as well as the highest loading accessory genes. The organization and gene content of these blocks is shown in Figure 1e and Supplementary Figure 8.

Block 1 (Supplementary Figure 8a & b), which has the most extreme loading variants, includes two genes with SNPs (KP1_0337 & KP1_0338). These genes are part of the five-gene *Kpa* fimbriae locus, which is highly conserved in both KP and *K. variicola*[27,28]. While the *Kpa* fimbriae are less characterized than the *fim* locus, experiments in LM21 (a low-loading strain) found that absence of *kpaC*, encoding the usher, significantly reduced *in vitro* biofilm formation in microtiter plates[29]. The Δ*kpaC* mutant did not affect binding to either a human cell line or *Arabidopsis* seedlings[29]. The most differentiated gene (KP1_0338/*kpaE*) encodes the adhesin subunit.

Block 2 (Supplementary Figure 8c & d) and Block 4 (Supplementary Figure 8g & h) also include SNPs in single genes (KP1_1632, filamentous haemagglutinin N-terminal domain-containing protein and KP1_5387, winged helix-turn-helix transcriptional regulator, respectively). The former, as for the *Kpa* fimbral adhesin, suggests a difference in adhesion, but so far appears uncharacterized. The regulator encoded by KP1_5387 is also unstudied, but seems always encoded next to an alcohol dehydrogenase in KP, *K. variicola*, *K. quasipneumoniae*, *K. africana*, *K. quasivariicola* strains.

Block 3 (Supplementary Figure 8e & f) includes both SNPs and accessory genes and can be subdivided into three sub-blocks (3a, 3b, 3c) based on LD patterns. The majority of strains fall into two configurations (Supplementary Figure 8f), with the high loading configuration including all accessory gene elements and the low loading configuration including none, but there are also strains with intermediate configurations.

Block 3 occurs in two different regions of the genome in the reference strain, with block 3b occurring in a different genome region from blocks 3a and 3c. Blocks 3a and 3c were

previously highlighted as associated with invasive strains[30], as well as those implicated in bovine mastitis[31]. Block 3a includes SNPs in two genes, one of which is a c-di-GMP-related phosphodiesterase encoded by all KP. Key genes of 3a are *kfuABC*, encoding a ferric iron ABC transporter, which aids iron acquisition and impact mouse colonization[30,32]. The block also encodes two c-di-GMP-related proteins, a regulator, and glycogen/PTS sugar utilization/uptake genes. Block 3c occurs adjacent to Block 3a in the genome and contains genes related to sugar uptake, metabolism, and regulation (again via c-di-GMP). Block 3b contains accessory genes encoding a cellulose synthase, three DUF3131 proteins, OpgC (osmoregulated glucan production), and several regulatory proteins (hybrid sensor kinase, anti-sigma-factor and its antagonist).

**Accessory but not core genes show evidence for functional enrichment of differentiated genes**

We compared the distribution of PC1 loading values between core SNPs and accessory genes, and found a clear difference between the two. The distribution of core SNPs was broader with longer tails, while that of accessory genes was more sharply peaked and centered around zero (Supplementary Figure 9a).

To establish whether there is a systematic relationship between gene function and SNP loadings, we calculated the mean absolute SNP loading for genes in each COG functional category (Supplementary Figure 9b) and found almost no difference between categories. However, larger and statistically significant differenced were found for accessory genes (Supplementary Figure 9c), which likely reflect that accessory genes are on average more strongly associated with PC1 (Supplementary Figure 9c).

**Virulence, antibiotic and isolation source profiles provide few clues**

Although virulence and antibiotic resistance are important for the clinical assessment of KP, our analysis suggests that they are not the primary determinants of its overall population structure. Fig. 1b shows the isolation metadata and Kleborate[24]-assigned resistance and virulence scores for the non-redundant dataset. Strains do not cluster based on geography, resistance, or virulence.

For multi-host bacteria, host-imposed selective pressures are important drivers of their evolution[33,34]. KP can be isolated from a variety of sources, including animals, plants, humans (which represent the largest proportion in databases), and water, including the sea. However, there is no strong evidence to support that host selective pressure is responsible for shaping the backbone of the KP phylogenetic tree.

Based on KP1_0338, the gene most strongly associated with PC1, we classified the multi-source strains into high and low variant groups (Supplementary Figure 10a). A Fisher's exact test revealed a significant association between KP1_0338 variant group and host source ($p < 0.01$), primarily driven by strains from water and horse (Table 1). However, we found that the most water-isolated strains were from Manchester, where they were collected from patient or hospital wastewater sources. According to Kleborate annotation, most of these strains had a resistance score of 2 and a virulence score of 0 or 1, indicating that they are typical hospital-associated clones. We think this signal as an artifact of the strains mostly belonging to a couple of clonal lineages rather than evidence of true host specificity.

| **Source** | **High** | **Low** | ***p*-value** |
|---|---|---|---|
| Animal | 10 | 22 | 0.410 |
| Bird | 6 | 13 | 0.593 |
| Cat | 8 | 34 | 0.472 |
| Chicken | 8 | 53 | 0.0341 |
| Cow | 129 | 377 | 0.676 |
| Dog | 26 | 74 | 0.812 |
| Environment | 52 | 198 | 0.136 |
| Food | 11 | 20 | 0.206 |
| Horse | 53 | 67 | 0.00000180 |
| Human | 147 | 410 | 0.324 |
| Mouse | 1 | 16 | 0.0891 |
| Pig | 27 | 111 | 0.153 |
| Water | 8 | 79 | 0.000307 |

Table 1. Enrichment of KP1_0338 gene types among different host sources based on Fisher's exact test.

For each host, a one-vs-rest Fisher's exact test was performed to evaluate whether the gene type distribution differed significantly from that of all other sources combined.

The KP isolates from horses were predominantly collected in France and Iceland, from various anatomical sites through both post-mortem examinations and live-animal sampling. But when we projected the combined dataset - including the non-redundant dataset, along with strains isolated from water and horse sources - onto the results corresponding to Fig. 1b (Supplementary Figure 10b), the projection reveals that the distribution of horse-derived strains was not significantly different from that of the non-redundant dataset. This is because, in our host association analysis, all strains were simply categorized into high and low groups based on KP1_0338, while intermediate types were ignored.

Both versions of KP1_0338 can be found across all host sources, suggesting that the observed population structure of KP is not shaped by simple host-specific selective pressures. Moreover, no clear associations were observed between population structure and geographic origin, antimicrobial resistance, or virulence Instead, the distribution of this gene appears to be largely independent of host origin, indicating that other ecological or evolutionary factors may underlie the observed patterns.

**The backbone is not explained by simple neutral models**

The presence of this substantial component of variation begs the question: how has it arisen? Simple models of a bacterial population evolving with mutation and homologous recombination failed to recapitulate the patterns we observed in KP. Simulations with a population size of 2000 and one homologous recombination event per genome of size 10 kb approximately matched the overall LD (Linkage Disequilibrium) decay curve seen in the real data (Figure 2a). However, for these parameters, there was no phylogenetic backbone evident, and PC1 failed to explain as much variance as in the real data (Figure 2b & c). As noted above, PC1 was also not reproducible when applied to different halves of the tree in a half matching test (Supplementary Figure 4). More generally, we have never seen a phylogenetic backbone in any simulations of a single neutrally evolving population (*e.g.*, Supplementary Figure 11).

The model of evolution shown in Figure 2B assumes that recombination rates are uniform across the species. More complex models are possible, for example in which recombination rates are high between closely related strains but lower between more distant ones. Such a pattern can arise for example if homologous recombination is suppressed by mismatches between donor and recipient sequences[35]. Such models are thought to explain much higher recombination rates within *Escherichia coli* phylogroups than between them[36]. Many models with these properties are possible, but since they all reduce the effectiveness of recombination, they are likely to make it difficult to recapitulate the low levels of LD in

KP at large genetic distances. Moreover, the models that have been investigated previously cause species to cluster into discrete groups[37,38], rather than generating an approximately continuous pattern of variation. We are unaware of any model in which variable recombination rates within a single population predict a continuous structure similar to that seen in KP (Supplementary Figures 11 & 12).

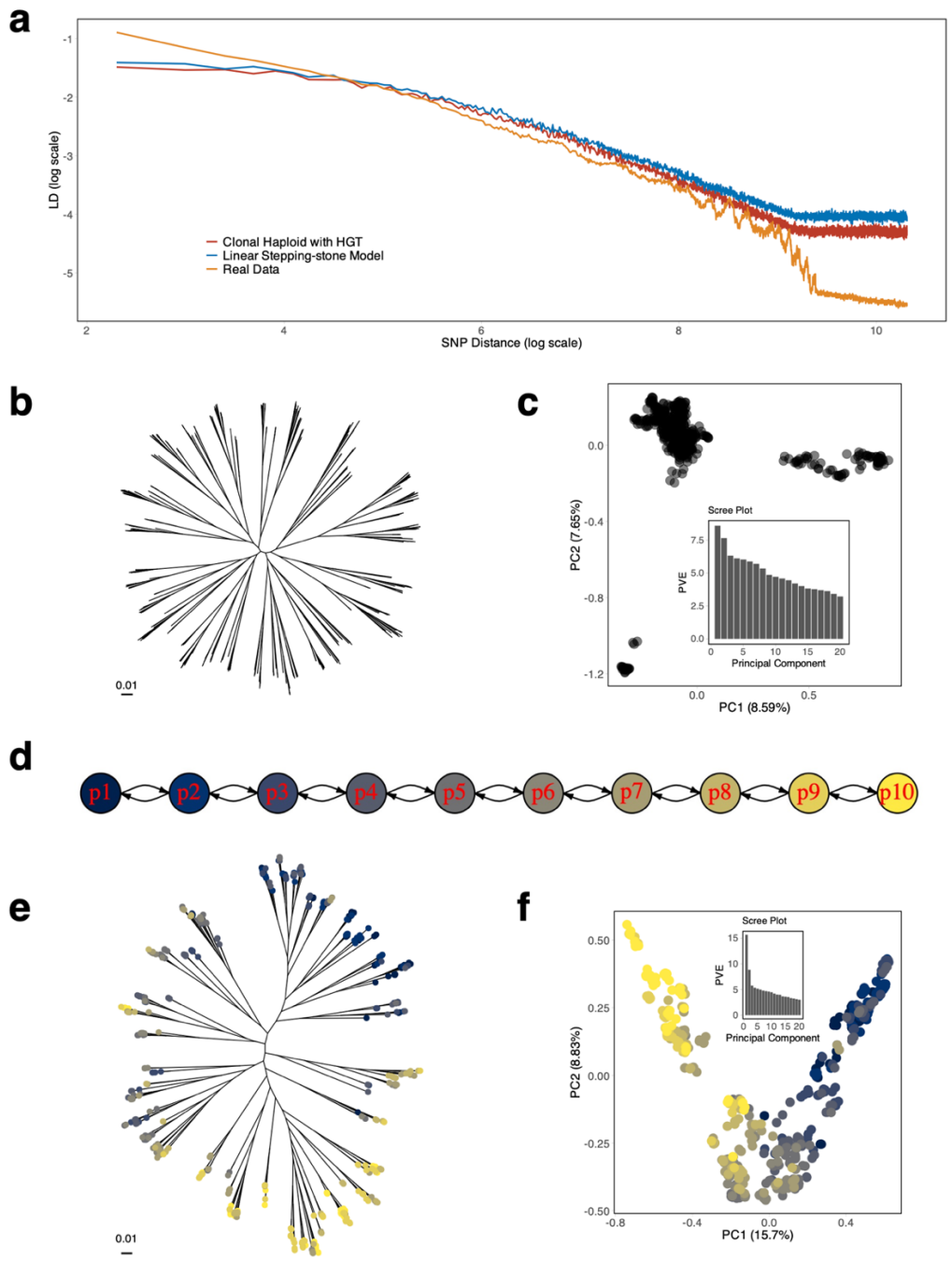


**Figure 2.** Simulated neutral models.

(a) Linkage disequilibrium (LD) decay comparison to real data for best-fit neutral single population and 10 population stepping-stone models. Phylogenetic tree (b) and PCA results (c) for single population evolving neutrally. (d) Illustration of 10 population stepping-stone model. Phylogenetic tree (e) and PCA (f) for best fit stepping-stone model, coloured as shown in (d).

Genetic differentiation can arise due to geographical structure, or other factors that restrict gene flow between particular subsets of the population19,39,40. To explore the properties of geographical population structure we next simulated a stepping-stone model with 10 demes (Figure 2d). The demes are organised along a line, with a small number of migrants moving between adjacent demes each generation. Data simulated according to this model gave a tree with a phylogenetic backbone similar to that seen in KP, with PC1 explaining a similar fraction of the overall variation (Figure 2e & f and Supplementary Figure 13) and also approximately matched the LD decay curve (Figure 2a and Supplementary Figure 14). However, the distribution of the SNP loadings for PC1 for these simulations did not match the real data, because there were many more SNPs with intermediate loading values (Supplementary Figure 15). Geographic population structure creates differentiation between populations through genetic drift, which is a random process that affects all of the genome but to differing degrees. The discrepancy in SNP loading values between simulated and real data implies that the variation in differentiation values in KP is higher than expected under a pure random drift model.

A longer-tailed distribution of drift levels, and hence PC1 values, could be generated by natural selection at a fraction of loci associated with adaptation to specific demes. However, even if a geographic model with some selection could be made to fit the data, this model seems to be poorly motivated in the case of KP. There is no evidence of KP being structured geographically (Figure 1b)[10,18] or of there being barriers to gene flow between ecological demes, since for example it is readily acquired by humans from a wide variety of ecological sources[41,42]. It is therefore hard to see how a model based on strong isolation between demes could be applicable to the species.

**KP variation is consistent with diversifying selection on a quantitative trait**

Many traits are subject to diversifying selection[43]. For example, the long necks of giraffes allow them to reach leaves that are high up in trees but are costly to grow and maintain. In a population of giraffes, the individuals with the longest and shortest necks might therefore have a fitness advantage over intermediate individuals. The individuals with the longest necks can reach leaves that other individuals cannot, while those with the shortest necks need to eat less leaves to maintain themselves[44].

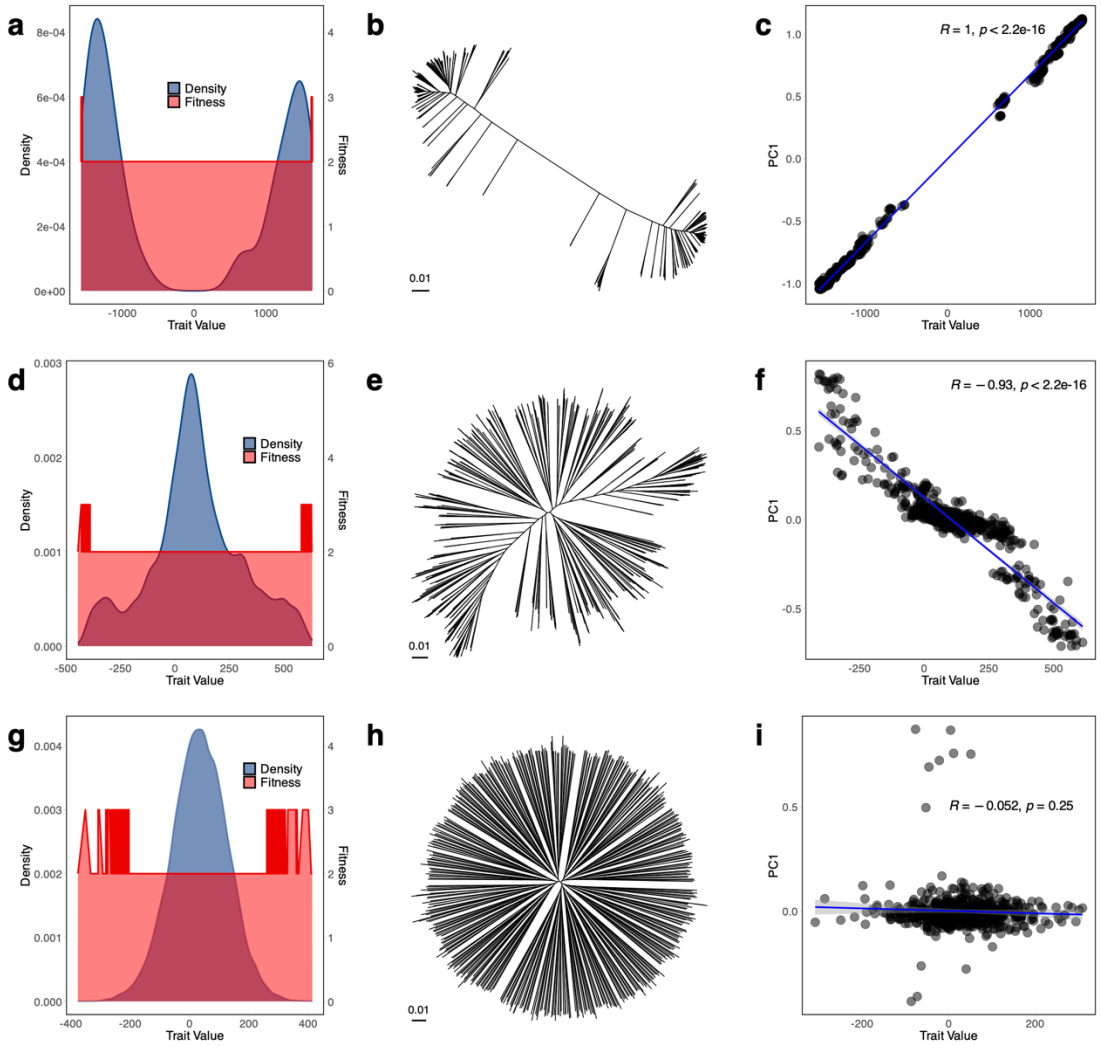


**Figure 3.** Simulated model of diversifying selection on a polygenic trait shows population structure depends on the balance between selection and recombination (1 Mb). (a, d, g) Trait value density distributions and fitness coefficients for HGT lengths of 1 kb, 10 kb, and 100 kb (Top to bottom) in final generation of simulation. (b, e, h) Phylogenetic trees depicting evolutionary relationships under each HGT length. (c, f, i) Correlations between PC1 values and trait values under each HGT length.

We have performed simulations of selection on a single quantitative trait in a recombining bacterial population. In the first simulations, each new mutation had a consistent effect on the magnitude of the trait, with half of all mutations decreasing it by one and half increasing it by one. We assumed that fitness is the same for all values of the trait, except that the bacteria with the highest and lowest values of the trait have a fitness advantage (Figure 3a, d and g). Simulation results showed that low recombination rates allow progressive divergence in trait values (Figure 3b), while high rates homogenise the population, leading

to a circular star-shaped phylogeny (Figure 3h). At intermediate recombination values, a phylogenetic backbone appeared similar to the one seen in the KP data (Figure 1a). For low and intermediate recombination rates (1 kb, 10 kb), PC1 values were strongly correlated with the true simulated trait values (Figure 3c, f and i, Supplementary Figure 16).

These simulations failed to recapitulate the sharp peaks in SNP loadings seen in the KP data (Supplementary Figure 17 vs. Figure 1c). We therefore modified the simulations, by assuming that the distribution of the magnitudes of the effect of SNPs on the trait followed a gamma distribution (Supplementary Figure 18). High values of the gamma parameter correspond to some mutations having a much larger effect on the trait than others.

Based on simulations with a 1MB chromosome length (Supplementary Figure 18), we found that the parameters of the gamma distribution had little impact on the structure of the phylogenetic tree and the results of the PCA. However, the parameters determining the SNP coefficients had a significant influence on the distribution of trait values and the distribution of SNP loading in the population. In real data, only a few peaks are observed, which might imply that only a small number of loci strongly influence the selected trait, while other loci, either do not contribute at all or have much smaller effects.

We found that a reasonable qualitative fit to the KP data could be found by simulating a 5 Mb genome length (similar to the true length), assuming trait values for each SNP followed a gamma distribution with a mean of 1 and a scale parameter of 5×106 (shape of gamma distribution = 2×10-7) (Figure 4). Specifically, we were able to recapitulate a phylogenetic backbone, a PC1 correlated with the trait value and a small number of high loading peaks (Figure 4c; Supplementary Figure 19), each of which is associated with SNPs with substantial influence on trait values.

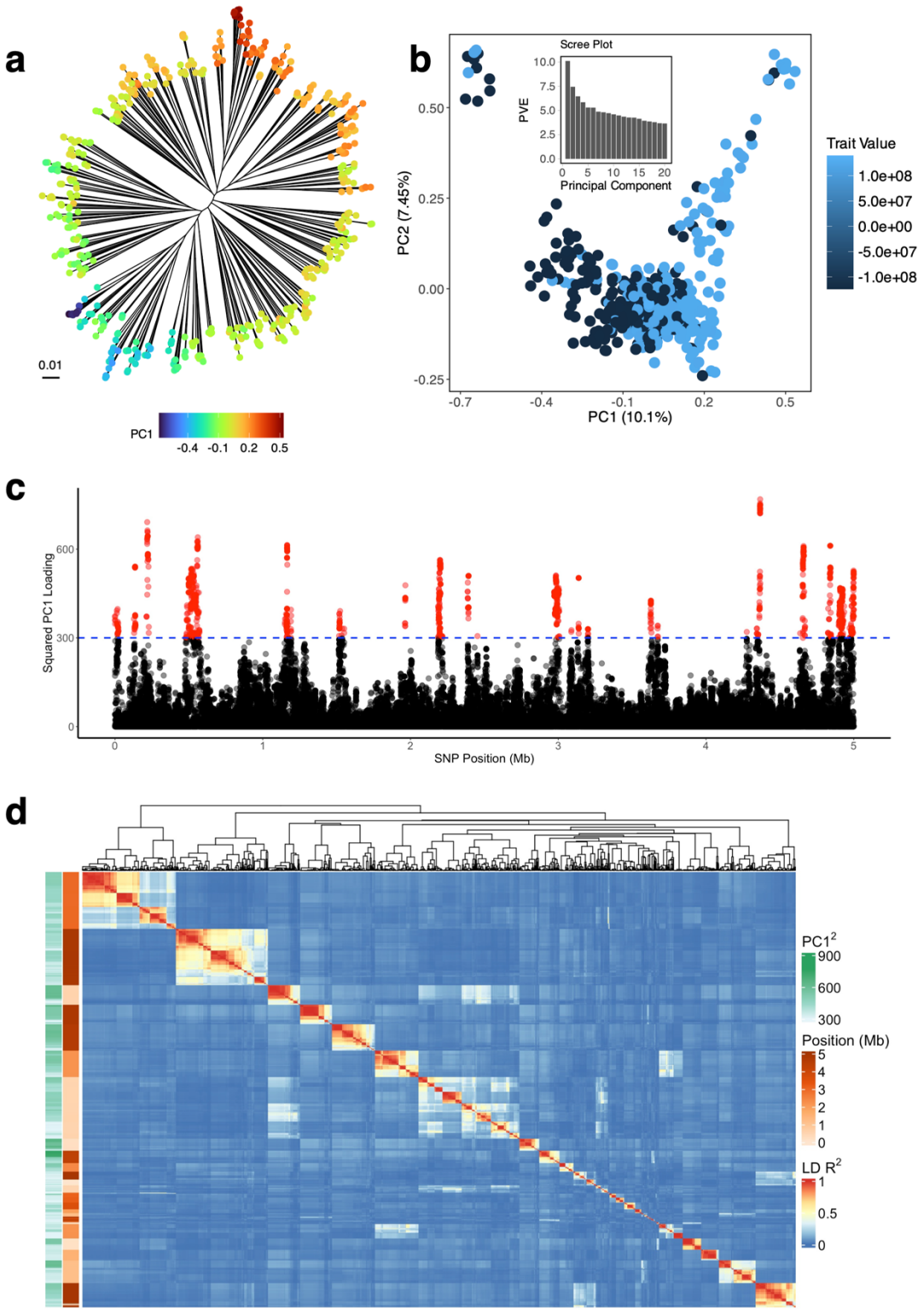


**Figure 4.** Best fit simulated diversifying selection model (5 Mb).

(a) Phylogenetic tree. (b) PCA results. (c) Squared SNP loadings on PC1 using the same threshold with

Fig 1c. (d) Heatmap of pairwise LD $R^2$ values for high loading SNPs.

## Discussion

### Do bacteria have quantitative traits?

Quantitative trait models are central to our understanding of human disease genetics[45] and animal[46] and plant[47] biology. These models are applicable to many morphological and disease susceptibility traits, which fit the assumptions of the model, namely that the genetic basis of variation of the trait in the population is highly polygenic, with most variants acting approximately independently of each other in their influence on the trait value[48,49]. These features allow the traits of offspring to be predicted based on the values found in their parents. The predictability inherent in these models has found several compelling practical applications, including predicting disease risk in humans, using Polygenic Risk Scores[50] and to assess the genetic quality of livestock, based on estimated breeding values[51]. The same rules also make short-term evolution relatively predictable[52]. We could for example use quantitative genetics to predict the change in giraffe neck length in a given population over a few generations of artificial selection, based on the intensity of selection and the additive genetic variation present within the population before selection takes place[53].

Bacteria do not undergo sexual reproduction in each generation, but instead reproduce clonally, while occasionally importing tracts of DNA from other strains[54]. The population-level response to selection depends on specific lineages rising in frequency[55]. As a result, the most straightforward applications of quantitative trait models in eukaryotes, such as predicting offspring trait values do not apply[56]. Despite these differences, bacteria do have many continuous valued traits[57], such as cell size, growth rate and antibiotic Minimal Inhibitory Concentration (MIC). It is of interest to understand the genetic variation of each trait circulating within species and how the trait responds to selection.

Laboratory selection experiments have provided important evidence on the genetic architecture of the response to selection in a novel environment[58]. Bacterial fitness in a particular environment is a continuous valued trait[59]. In asexual laboratory populations, mutations of large effect dominate the initial response, but diminishing returns epistasis sets in rapidly with later variants having progressively smaller effect[60]. Although these laboratory experiments provide excellent evidence of short and medium term responses to novel environments, we currently have neither data nor theoretical models applicable to natural recombining populations of bacteria. For example, if a lineage is selected for increased cell size, we do not know whether the response is likely to be driven by variants of small or large effect, and whether the variants will principally arise de novo or be

imported from other members of the population by homologous or non-homologous recombination.

To our knowledge, the only continuous valued trait whose variation has been studied extensively in bacterial populations is antibiotic MIC, which can be treated quantitively[61]. Antibiotic resistance is however an unusual trait, because of the episodic nature of exposure to antibiotics and the strong selection coefficients involved, which accentuates the role of variants of large effect[62]. The most important sources of variation are mobile genetic elements carrying antibiotic resistance islands and de novo point mutations. These variants do not fit the assumptions of quantitative trait models because individual variants have large effect and moreover, there are strong epistatic interactions between them[63,64]. Furthermore, when antibiotic selection pressure is applied, instead of increasing MIC across the population, the outcome is some lineages become highly resistant, while others remain susceptible[65].

One factor that is likely to be critical in determining the genetic architecture of a trait is the past selection history[66]. For example, if cell size has previously been consistently selected to be smaller within the natural environment, then we might expect the response to selection for larger cell size to be like that seen in laboratory selection environments, with de novo mutation dominating and the initial response generated by mutations of large effect. By contrast, if there has been fluctuating or diversifying selection in the population, then many variants affecting size may already be segregating. These existing variants will have already been tested by selection and will tend to be less disruptive of other features of the organism's biology. Therefore, if we want to find traits that are polygenic within bacterial populations and that variants might behave additively, with most variants having small effect, we might focus on traits that have been under such selection regimes.

Here we have asked whether there are circumstances in which selection on a quantitative trait might structure the genetic variation across an entire bacterial species. For example, episodic antibiotic selection structures bacterial population into resistant and sensitive strains, which have higher fitness in environments with and without antibiotics[67]. Since humans only started applying antibiotics a few decades ago, the typical signature of this selection is clonal expansion of antibiotic resistant lineages[68]. We can imagine that other traits, such as cell size, might be more polygenic and might have been under fluctuating diversifying selection, favoring a mix of high and low trait values, for longer periods of time. In this case, the selection might leave a more dispersed but still distinctive genetic signature.

Specifically, we have performed simulations of an additive trait in which strains with the highest and lowest values of the trait have a small fitness advantage. However, this tendency is counteracted by recombination, which randomises variation within the population. If the recombination rates at each locus are low, strains are likely to differentiate into two discrete groups with distinct trait values (Figure 3a). If recombination rates are higher, then intermediate strains can become common (Figure 3b & c). Nevertheless, selection for extreme values ensures that variants which increase trait values will be found together more often than expected under random assortment. These non-random associations represent a form of linkage disequilibrium, which can be detected with a variety of statistical approaches.

One approach to detecting non-random associations between variants is PCA, which is particularly suited to identifying factors that act genome-wide such as population structure[69,70]. When we perform PCA on a simulated population under diversifying selection, the values of the first component, PC1, for each strain can be strongly correlated with trait values (Figure 3c, f and i, Supplementary Figure 16). We call the gradient in PC1 values a bacterial ecocline, since it does not require geographical or other barriers to gene flow and is generated simply by natural selection on a trait, which in real populations is likely to generated by ecological factors.

The clinal variation we see in our simulations depends on a balance between selection and recombination. As such it is unlikely to arise in outbreeding animals and plants. In these species chromosomal segregation randomizes variants each generation, making recombination a stronger force than selection. As such, a bacterial ecocline is a novel form of genetic structure. The term ecocline is also used by eukaryotic biologists[71,72]. In their usage, the term describes a situation where there is a single key ecological variable, such as altitude above sea levels for terrestrial animals or saltwater concentration for marine ones, which generates a strong geographical gene frequency cline in the loci that determine adaptation to that variable[72]. However, in their context, the gene frequency cline is dependent on limited dispersal between locations, which reduces the rate of recombination between individuals with high and low trait values. We think that our use of the term ecocline is more accurate, while theirs should strictly be called geo-ecoclines. Their clines are however like the one we have simulated in being dependent on a key ecological trait.

**Reverse ecology of bacterial ecocline**

We do not yet have compelling evidence for any bacterial ecocline. To identify and understand them biologically, we propose a reverse ecology approach[73], in which we first

identify that variation within a species is organized into a putative ecocline and then proceed to establish the phenotypic trait or traits that might underly that cline based on functional annotation and laboratory experiments, before finally attempting to understand its ecological significance and what maintains variation in the population. This approach entails making an ecological hypothesis - that diversifying selection on a trait has generated clinal variation - and a functional hypothesis - that the genetic variants showing clinal variation are modifying the selected trait. Demonstration of an ecocline requires validation of both hypotheses.

As our application to simulated data demonstrates, PCA is a potentially a useful tool for detecting possible bacterial ecoclines. However, considerable caution must be exercised in interpreting its results, because PCA will detect apparently clinal variation in essentially any dataset. The PC1 cline in KP is noteworthy because of the large fraction of variation it explains (Figure 1b), its reproducibility in analyses using subsets of the data (Supplementary Figure 3) and the difficulty in explaining these features using other models for generating genetic variation. Specifically, we have been unable to obtain similar patterns using simulated neutral models (Figure 2), with or without geographic structure. Models with geographical barriers to gene flow and geographical variation in selection coefficients might be able to generate an ecocline-like structure but they would also entail geographical differentiation, which there is no trace of in KP.

Fortunately, we do not have to rely on PCA alone to visualize and interpret the clinal variation that we see. In the simulations that best fit the KP data most of the variation in trait values in the population is generated by less than 20 sites in the genome. When PCA is applied to this data, the sites with the largest effect on trait values have extreme PC1 loadings (Supplementary Figure 18b), implying that if selection on a trait has generated PC1 then we can potentially identify the variants in the genome that have the largest effect on trait values based on their extreme PC1 loadings. We can visualize the linkage disequilibrium between sites with extreme loading values (Figure 1d) and see if the patterns are consistent with what we see in simulated ecoclines (Figure 4d).

In our diversifying selection model, linkage disequilibrium between sites arises because of the tendency of sites that increase trait values to accumulate on the same genetic backgrounds. There are no direct fitness interactions between the sites themselves. If there are no other forces generating LD, then this model implies that the sites with the largest effect on trait values should be in the strongest disequilibrium with all other sites in the genome. In practice, there are other forces generating LD, specifically including genetic linkage between sites that are physically close together on the chromosome. This means

that in both simulated and real data, the genome is organized into physically linked LD blocks, which can be seen as red squares on the diagonal in both Figure 1d and Figure 4d.

Once the block-like structure of LD in the chromosome created by physical linkage is taken into account, the diversifying selection model implies that most of the remaining LD should generated by selection of the trait. In the KP data, Block 1 has the most extreme PC1 loadings, so that this block is predicted to have the largest effect on the putative quantitative trait and concordant with this prediction, it has consistently high pairwise LD with loci in other blocks, while blocks 5-44, which have lower loading values, have consistently low pairwise LD values with other blocks. The LD patterns that we see thus seem broadly consistent with selection being mediated by the effect on a trait, rather than pairwise interactions between loci, which would generate a more patchwork-quilt like pattern.

There is one feature of the real data which is not matched by the simulations, namely that the four genome blocks with the most extreme PC1 loadings are also the genome blocks with the largest number of variants, with Block 1 having more than 100 SNPs (these 139 SNPs are in two genes, which are the same as the accessory genes, Supplementary Figure 8a & b). We think that this difference reflects a biologically unrealistic feature of our simulations, namely that each variant is a single site acting independently. However, functionally important variation can require changes at multiple sites, which could be generated by long-term diversifying selection on the trait. For example, Block 1 encodes the fimbria locus (Supplementary Figure 8a & 8b). Within the KP population, there are two highly distinct haplotypes, which differ on average at 139 sites, which potentially encodes for a substantially different biological function. This would be impossible to achieve using a single SNP.

The functions of the genes in the top four blocks are not obviously related. With the most differentiated gene being the adhesin component of *Kpa* fimbriae, this suggests the trait may be related to adhesion/diffusion. This is also supported by differentiated of several proteins related to c-di-GMP metabolism, a second messenger is thought to signal/control trade-offs between sessile/surface and motile/free-living behaviours[74]. The accessory *kfuABC* locus suggests there is some involvement of iron acquisition[32]. While these genes were previously found to be over-represented in invasive clinical isolates and bovine mastitis strains[31], we could not find any evidence that the trait is directly and specifically involved in human infection. One thing that is clear is that virulent or resistant genotypes can assemble on all positions of the ecocline.

We also attempted to identify specific gene function categories that were particularly

associated with the backbone. For core genes, there is no difference between COG in mean absolute PC1 loading values, but for accessory genes, metabolic and cellular process associated genes have systematically higher absolute loadings than genes of unknown function or those associated with replication, recombination or transcription. Simulation results show that depending on parameter values, trait values and SNP loadings (Figure 3, Supplementary Figure 18b) may only be strongly correlated for variants with large effect on trait values, with other loci showing effectively neutral patterns. Our provisional interpretation is that while three of the four most strongly associated loci consist only of core genome SNPs, most other core genome SNPs might have limited influence on the putative trait, while the presence or absence of specific metabolic genes is likely to be more important.

Despite the provisional nature of the current evidence, we have been emboldened to propose KP variation might be organized into an ecocline based on our previous finding of "ecospecies" in two others bacterial genera[20,25]. An ecospecies is similar to an ecocline, in that genetic differentiation is maintained by selection, in the absence of other barriers to genetic exchange. However, in an ecocline strains can fall anywhere along a gradient, while each strain must belong to one ecospecies or another and there are no intermediates. Because of their discrete character, ecospecies represent a particularly striking pattern of variation, which seem inconsistent with neutral models. Differentiation between Hardy and Ubiquitous *Helicobacter pylori* is largely restricted to 100 genes, which each have nearly fixed differences in at least 5 SNP and there are no intermediate genotypes[25]. In *V. parahaemolyticus* differentiation is restricted to at least 27 core genes, and once again there are no intermediate genotypes[20].

For ecospecies, we have also been able to make stronger functional predictions. Initial characterization of ecospecies-specific genes led to the hypotheses that the *V. parahaemolyticus* ecospecies were related to swarming behavior (lateral flagella) while the *Helicobacter* ecospecies are related to host diet (metal uptake genes) and interaction with the host (outer membrane proteins)[17]. Furthermore, the Hardy ecospecies of *H. pylori* has a lineage that has made a host jump to big cats but has only been isolated from humans who have historically had little access to plant matter in their diet, providing an immediate functional hypothesis: that the Hardy ecospecies survives better in the stomachs of carnivores[23]. Further evidence for this hypothesis is that Hardy strains carry an additional urease gene, cofactored by iron instead of nickel, which is otherwise restricted to *Helicobacter* species from carnivorous mammals[25].

The absence of equivalent functional predictions means that our current designation of a

bacterial ecocline in KP needs to be validated by phenotypic data. If the ecological hypothesis is correct, and the genes with extreme loadings are influencing a single trait, then characterization of the effects of these variants should simultaneously provide considerable insight into gene function, ecology and evolution for a key bacterial fitness trait. This would likely represent a breakthrough as these aspects are hard to study individually, handsomely justifying the initial effort required to identify the trait.

It should be cautioned however that diversifying selection may not reflect a single measurable trait. An alternative is that there is some form of evolutionary trade off driving selection. For example, giraffes could reach leaves high up leaves in trees either by growing a long neck or by being able to jump high. Giraffes with longer necks are likely to be particularly unsuited to jumping, so that selection favoring either solution but not both could create linkage disequilibrium between jumping genes and neck length genes[44]. In this scenario, there might be a single ecocline generated, which did not correspond to any single feature of giraffe morphology. Further, even if there is a simple trait in KP that is under diversifying selection, it might not be readily recreated under laboratory conditions.

A challenge and virtue of the reverse ecology approach is that it confronts us with the gulf between laboratory conditions and the natural environment that we seek to understand, and more specifically our difficulty in putting ourselves in the same shoes as bacteria and predicting the ecological challenges they are likely to face. In any case, we propose that investigation of phenotypic differences associated with the PC1 cline has the potential to deliver key insights into KP ecology, as well as on the biology of quantitative traits in microbes.

## Methods

### Genome collection and Sequence alignment

We collected 1,421 whole genome sequences of *Klebsiella pneumoniae* from the NCBI database, selected based on the test dataset for iterative-PopPUNK[76], along with a reference genome (*K. pneumoniae* strain NTUH-K2044, NC_012731.1). The reference genome is a well-characterized hypervirulent strain associated with liver abscess and meningitis[77]. Additionally, we obtained genome metadata using the NCBI Datasets command-line tools (v16.12.1)[78] and used Kleborate (v2.4.0)[24] to extract clinically-relevant genotypic information on strains (Table S1). All genomes were aligned to the NTUH-K2044 (NC_012731.1) reference genome using Nucmer (v3.1)[61] for reference-based alignment.

In total, 8,331 global *Vibrio parahaemolyticus* genomes (Table S2) were downloaded from the NCBI database, based on the dataset from the study 'Why panmictic bacteria are rare'[79] and aligned to the *V. parahaemolyticus* RIMD 2210633 reference genome (RefSeq NC_004603.1 and NC_004605.1)[80] using Nucmer (v3.1)[81], following the same alignment strategy described for *K. pneumoniae* and consistent with the approach described in previous *V. parahaemolyticus* studies[19,20].

### Core genomic variant calling and Definition of Non-redundant Dataset

SNP-sites (v2.5.2)[82] was used to call all the variants from the whole *K. pneumoniae* dataset alignment. Core SNPs were defined as the SNPs present in at least 99% of the genomes across the whole genome alignment, resulting in 850,158 core SNPs after extraction. Using these core SNPs, pairwise distances were calculated across all genomes with snp-dists (v0.8.2)[83]. To define a non-redundant dataset, genomes with pairwise SNP distances greater than 20 kb were selected, resulting in 369 non-redundant genomes (Supplementary Figure 1a).

We applied the same method to the whole *V. parahaemolyticus* dataset. Genomes with pairwise SNP distances greater than 50 kb were considered non-redundant, a threshold chosen to significantly shrink the dataset, resulting in a final dataset of 667 *V. parahaemolyticus* genomes with 1,619,597 core SNPs.

### fineSTRUCTURE and Population assignment

To further investigate the population structure of the non-redundant 369 *K. pneumoniae* strains described above, we performed chromosome painting and fineSTRUCTURE analysis. Using ChromoPainter mode in fineSTRUCTURE (v4.1.0)[84], we inferred chunks

of DNA donated from a donor to a recipient for each recipient haplotype and summarized the results into a co-ancestry matrix. Subsequently, fineSTRUCTURE clustered 369 individuals based on the co-ancestry matrix, with 100,000 iterations for both burn-in and Markov Chain Monte Carlo (MCMC) (Supplementary Figure 1b). In this analysis, only variants present in all non-redundant strains were considered, as fineSTRUCTURE requires input data without missing values.

To assign a population to the non-redundant *V. parahaemolyticus* dataset, we followed the same fineSTRUCTURE approach using haplotype data from core SNPs. Based on the fineSTRUCTURE results (Supplementary Figure 5a), the genomes were classified into five populations: VppAsia (506 strains), Hybrid (5 strains), VppUS1 (49 strains), VppUS2 (23 strains), and VppX (84 strains). Within the VppAsia dataset, strains were further categorized into ecospecies types (EG1a and non-EG1a) based on 27 differentiating genes[20].

**Construction of the Phylogenetic Tree**

Based on the core SNPs defined above, we constructed nucleotide sequence alignment files for both the entire *K. pneumoniae* dataset and the non-redundant *K. pneumoniae* dataset. Phylogenetic trees were constructed using FastTree (v2.1.10)[85] with the "-nt" option under the default model. The trees were visualized and compared using the ggtree package (v3.12.0)[86] in R, highlighting the phylogenetic relationships within the whole *K. pneumoniae* dataset (Supplementary Figure 2) and the non-redundant dataset (Figure 1a).

Similarly, we used the same software and parameters to construct phylogenetic trees for the non-redundant *V. parahaemolyticus* (Supplementary Figure 5b) and VppAsia (Supplementary Figure 6a) datasets, as well as the simulated datasets. During Partial-Variants Matching, we also constructed phylogenetic trees based on a subset of SNPs from the real datasets (*K. pneumoniae* and *V. parahaemolyticus*).

**Principal Component Analysis**

Before performing PCA (Principal Component Analysis) on the non-redundant *K. pneumoniae* dataset, PLINK (v1.9)[87] was used to filter out loci with a minimum allele frequency (MAF) below 2% from the core SNPs, leaving 102,226 variants. Linkage disequilibrium (LD) pruning was subsequently performed to remove linked SNPs, where one SNP from each pair was removed if the LD ($R^2$) exceeded 0.1 within a 50 kb window. The window was then shifted forward by 10 bp, and the procedure repeated to ensure comprehensive coverage. A total of 33,589 pruned variants were obtained and used for

conducting PCA on the non-redundant dataset with PLINK[87], extracting and reporting the first 20 principal components by default.

Similarly, we used the same software and parameters to perform PCA on the non-redundant *V. parahaemolyticus* dataset, which included 220,737 SNPs after MAF filtering and 69,537 SNPs after pruning. We also applied PCA to the VppAsia dataset, containing 207,292 SNPs after MAF filtering and 65,058 SNPs after pruning. For the simulated datasets, we only applied MAF filtering to retain enough SNPs, followed directly by PCA without pruning.

**Variant loadings calculation**

We first calculated the correlation between the original variables and the principal components, which is specifically referred to as the PCA loading for strains. The loadings can be determined as the coordinates of the variables divided by the square root of the eigenvalue associated with that principal component (Equation 1). When performing PCA, PLINK[87] generates output files ending with eigenval and eigenvec. Next, we transform the MAF-filtered SNP matrix into a binary matrix, assigning a value of 1 to minor alleles and 0 to all others (including the third and fourth most frequent alleles at non-biallelic sites). Finally, through matrix multiplication, we computed the product of the core SNP matrix and the PCA loading matrix to obtain the loading values for the MAF-filtered SNPs (Equation 2).

$$strain\ loadings = eigen\ vectors \cdot \sqrt{eigen\ values} \quad \text{(Equation 1)}$$

$$SNP\ loadings = strain\ loadings \cdot SNP\ matrix \quad \text{(Equation 2)}$$

To better highlight the significant peaks in the non-redundant *K. pneumoniae* result, we plotted the squared PC1 SNP loading values and marked the dots with squared loading values greater than 300 (Figure 1C, left). Supplementary Figure 3a shows the original SNP loading values for PC1, and Supplementary Figure 3b presents the zoomed-in views of the four peak regions with locus tags.

Similarly, we used the same method to calculate SNP loadings for the non-redundant *V. parahaemolyticus* dataset, the VppAsia dataset, and the simulated datasets.

**Pangenome analysis**

To estimate the gene content, we annotated all *K. pneumoniae* genomes with prokka (v1.13)[88] software, using a curated version of the NC_012731.1 (NTUH_K2044) proteome as the primary annotation source. The Generic Feature Format (GFF) outputs generated by

prokka were then used as the input files for panaroo (v1.2.8)[26] with the strict model. The "--remove-invalid-genes" and "--merge_paralogs" options were also employed during the analysis. Next, we extracted the non-redundant dataset from the overall results and defined 5,558 accessory genes as those present in between 2% and 98% of the genomes in the non-redundant dataset. Using the same approach (Equation 2), we also calculated the gene loadings and defined genes with squared PC1 loadings greater than 300 as high-loading genes (Figure 1C, *right*).

**Calculation of Linkage Disequilibrium Decay**

To analyze the decay of linkage disequilibrium (LD) in *K. pneumoniae*, we included 102,226 MAF-filtered SNPs and used the Haploview (v4.2)[89] software to compute $R^2$ values. When running Haploview, we set "-maxdistance 30" to ensure that the maximum marker distance for $R^2$ calculation did not exceed 30 kb. Additionally, we set "-minMAF 0" and "-hwcutoff 0" to exclude any filtering based on minor allele frequency and Hardy-Weinberg equilibrium testing, ensuring that all input SNPs were included in the calculations. The average $r^2$ value was calculated using a 10 bp step size.

Similarly, the same software and parameters were used for the simulated datasets to obtain the LD decay results for the simulated data.

We also calculated the LD between the high-loading variants defined above (340 variants in total, including core SNPs and accessory genes) used Equation 3 in R. Each element $R_{ij}^2$ of the matrix quantifies the degree of linkage disequilibrium between variant *i* and variant *j*, providing a comprehensive view of the genomic associations among core SNPs and accessory genes. Based on manual inspection, we removed an accessory gene (group_205) that represented fragmented versions of KP1_1982 (339 variants left), which is visualized in the heatmap Figure 1D, generated using the ComplexHeatmap package[90].

$$R^2 = \frac{\left(f_{gene_i,gene_j} - f_{gene_i} f_{gene_j}\right)^2}{f_{gene_i} f_{gene_i'} f_{gene_j} f_{gene_j'}} \qquad \text{(Equation 3)}$$

Based on the LD heatmap of all high-loading variants, we performed hierarchical clustering to divide the heatmap into 44 blocks. Among these, 4 larger blocks contain multiple variants, while the remaining 40 smaller blocks each contain only a few variants. The details are annotated in Figure 1D. Notably, block 3 was further subdivided into 3a, 3b, and 3c based on the physical locations of the mutations on the reference genome. We observed that some SNPs in block 3 are located within the KP1_RS30360 and KP1_RS30365 genes

in the reference genome. According to the annotation file of the reference genome, KP1_RS30365 is classified as a pseudogene. However, we do not concur with this interpretation. We believe that KP1_RS30360 and KP1_RS30365 together represent a complete gene, with some strains (*e.g.*, MGH 78578) containing only a fragment of this gene. the differentiation at this locus also likely represents a functional presence/absence rather than functional differentiation via SNPs.

As mentioned above, we converted the variants into binary, where 0 indicates that the core SNP is a major SNP and the accessory gene is absent in the strain, and 1 indicates that the core SNP is a minor SNP, and the accessory gene is present in the strain. We then generated heatmaps of the variant states according to the blocks, with rows following the same order as the LD heatmap and columns subjected to hierarchical clustering. The details are shown in the supplementary figure 4.

**Functional Annotation**

To further characterize the functional content in *K. pneumoniae*, we annotated both the MAF-filtered SNPs (MAF > 0.02) with SnpEff (v4.3t)[91], using the reference genome NTUH-K2044 (NC_012731.1), and applied custom filtering to focus on coding region variants by disabling annotation for upstream, downstream, and UTR (untranslated) regions. We then obtained the types of the SNPs and determined whether they were synonymous mutations. For accessory genes, we used the eggNOG-mapper[92] online tool with its default settings to ensure a comprehensive and standardized annotation.

For the identified high-loading variants (squared PC1 SNP loading > 300), we further mapped these variants to the reference genomes of *K. pneumoniae* strains MGH 78578 (NC_009648.1) using BLASTn[93]. The mapping aimed to identify their genomic locations and potential functional implications in these strains. Additionally, we searched the UniProt[94] database to retrieve gene names and associated functional annotations for the high-loading variants.

We calculated the mean absolute PC1 loading for accessory genes within each COG category, as well as the overall mean absolute loading across all accessory genes. For core SNPs, we first computed the mean absolute PC1 loading of SNPs belonging to each core gene, then calculated the average of these values for each COG category. In addition, we determined the overall mean absolute loading across all core genes.

We visualized the results using bar plots, where each bar represents the mean absolute PC1 loading of genes (or SNPs) within a given COG category, and the error bars indicate the

standard error of the mean. A dashed line denotes the overall mean absolute PC1 loading across all accessory genes (or core genes), serving as a reference.

**Isolation background characteristics**

To investigate potential host specificity in KP, and to address the overrepresentation of human-derived isolates in our primary dataset, we incorporated a multi-host dataset from the Pasteur Institute Klebsiella MLST Database. Isolates were selected based on the following quality criteria: chromosomal assembly size between 5 and 6 Mb, fewer than 100 contigs, an N50 greater than 20,000 bp, fewer than 1,000 ambiguous bases (Ns), and a GC content between 55% and 60%. Additionally, only genomes with "good" annotation status for MLST, ribosomal MLST, and SCGMLST629_s schemes were included. Kleborate was applied to refine species classification, and *Klebsiella pneumoniae* species complex (KpSC) isolates were excluded.

We used the KP1_0338 gene from the reference strain as the query sequence and included MGH 78578 as an additional representative. These two strains represent the extremes along the PC1 axis in our population structure analysis, with the reference strain showing an extremely high PC1 value and MGH 78578 a low one, thus capturing the two major variants of KP1_0338 present in the KP population. Using BLASTn, we identified KP1_0338 homologs in the multi-host dataset and constructed a phylogenetic tree to examine sequence diversity. Based on the topology of the KP1_0338 phylogeny, isolates were grouped into two distinct clusters, designated as High and Low types.

To assess host-specific enrichment of these gene variants, we performed a one-vs-rest Fisher's exact test for each host. This test compared the counts of High and Low types within each host to those in all other hosts combined. The results are summarized in Table 1.

We projected the combined dataset—including the non-redundant dataset as well as strains isolated from water and horse sources—onto the principal component space defined by the non-redundant dataset. For each group (non-redundant, water-derived, and horse-derived), we calculated the mean PC1 loading values and visualized them with standard deviations as error bars. Pairwise comparisons between groups were performed using two-sample t-tests to assess the statistical significance of differences in PC1 loadings.

**Half-matching testing**

To assess the stability and repeatability of the first principal component (PC1) in *K.*

*pneumoniae*, we used three half-matching tests, each designed to evaluate the consistency of PC1 under varying conditions.

**1. Half-Strain Matching**

Firstly, we divided the non-redundant *K. pneumoniae* dataset into two groups based on the backbone in the phylogenetic tree (right-hand side and left-hand side). We then performed PCA on the first group using the pruned SNPs. Afterward, we calculated the MAF-filtered SNP loadings using the above steps. Subsequently, we obtained the product for the second group through matrix multiplication, representing the mapping of the PCA results from the first group onto the second group. We then calculated the correlation for the PC1 between the mapped results of the first group and the PCA results of the second group. Conversely, the same matching analysis was performed on the data from the second group.

**2. Half- Segment Matching**

*K. pneumoniae* has a circular chromosome with a length of approximately 5 to 6 Mb, and the reference genome we used is approximately 5.2 Mb in length. We divided the circular chromosome into two DNA fragments (Fragment 1 (1 bp–2.6 Mb) and Fragment 2 (2.6 Mb–5.2 Mb)), each containing approximately half of the chromosome. Specifically, the first dataset comprised the pruned SNPs in the first half of the chromosome, while the second dataset encompassed the unlinked SNPs from the second half. We separately performed PCA on these two datasets using PLINK. Finally, we calculated the correlation between the PC1 of the two fragments.

**3. Partial-Variants Matching**

First, we assigned maximum loading values to the MAF-filtered SNPs based on their squared PC1 loadings, using a 50 kb window size with a 1 bp step size. This process identified the maximum squared PC1 loadings within each 50 kb vicinity. Based on the distribution of maximum squared PC1 loadings, the SNPs were categorized into three types: low-loading SNPs, medium-loading SNPs, and high-loading SNPs (*e.g.*, Supplementary Figure 3g). We separately conducted PCA on the high-loading SNP dataset and low-loading SNP dataset, by extracting unlinked SNPs from each. Subsequently, we calculated the correlation between the PC1 of the two datasets. In this test, we ignored SNPs with intermediate loading values.

Similarly, the three types of half-matching tests were applied to the non-redundant clonal population simulation (Simulation 1, SNP distance > 2 kb, shown in Supplementary Figure

4), the non-redundant V. parahaemolyticus dataset (Supplementary Figure 5) and the VppAsia dataset (Supplementary Figure 6). However, in the half-strain matching test, although we still performed splits along the phylogenetic tree, these datasets were not always divided into left-hand and right-hand subsets in the trees. Therefore, in these datasets, we referred to the two subsets as half samples 1 and half samples 2. Additionally, in *V. parahaemolyticus*, which inherently possesses two chromosomes of different sizes, we directly divided the data into two parts based on chromosome numbers.

**Simulation of bacterial populations**

We used SLiM (v4.0.1)[95] to simulate the evolutionary dynamics of bacterial populations under different scenarios using a non-Wright–Fisher (nonWF) framework[96]. All scenarios started with identical initial conditions: each bacterial genome consisted of a single 1 Mb haploid chromosome, with a mutation rate of $10^{-6}$ per site per generation, approximately ten times higher than the estimated mutation rate for *K. pneumoniae*[97]. Horizontal gene transfer (HGT) occurred at a rate of 1.0, ensuring that every individual experienced HGT in each generation. The simulations ran for 10,000 generations. Two mutation types, "m1" and "m2", occurred at each site with equal probability (1:1) and were assumed to be neutral. However, in the final simulation, we adjusted the chromosome length to closely match the length of a real *K. pneumoniae* genome.

**Modeling clonal haploid bacteria with horizontal gene transfer**

In the first simulation scenario, we modeled the evolutionary dynamics of a single bacterial population, focusing on the spread of a single type of neutral mutation and the genetic exchange within population via HGT. The HGT tract length was set to 100 bp, 1 kb, 10 kb, or 100 kb, representing a range of recombination sizes to accurately capture genetic variation within the population. Each bacterium produced offspring according to a Poisson distribution with $\lambda = 2$, meaning that, on average, each bacterium produced two offspring per generation. However, the number of offspring per bacterium in any given generation could vary, with some bacteria producing more and others producing fewer, reflecting the probabilistic nature of the Poisson distribution. We conducted three trials with population sizes of 1,000, 2,000, and 10,000 (Supplementary Figure 11 & 12).

**A linear stepping-stone model**

In the second simulation scenario, we constructed a linear stepping-stone model with 10 subpopulations of clonal haploid bacteria, where each subpopulation was connected by migration only to its nearest neighbors in the chain. The average recombination tract length

within each subpopulation was set to 10 kb. Migration occurred with varying numbers of migrants - 1, 10, 20, or 50 individuals - moving to neighboring subpopulations each generation to simulate gene flow driven by geographical factor or other isolating barriers. Each individual produced offspring according to a Poisson distribution with $\lambda = 2$. We conducted three trials with total population sizes of 1,000, 2,000, and 10,000, corresponding to 100, 200, and 1,000 individuals per subpopulation, respectively (Supplementary Figure 13 & 14).

**Selective model**

In the third simulation scenario, we focused on a population of 10,000 clonal haploid bacteria, introducing selective pressure into the model. Horizontal gene transfer (HGT) tract lengths were set to 1 kb, 10 kb, and 100 kb. Each individual was assigned a score, termed a quantitative trait value, based on the difference in the number of "m1" and "m2" (score $= \sum \mathrm{m1} - \sum \mathrm{m2}$) and ranked accordingly. In this model, the coefficient for any locus on the chromosome was 1. Depending on the mutation type, if it was m1, the value at that locus was 1; if it was m2, the value at that locus became -1. From the top and bottom 160 individuals, 80 were selected to have increased offspring production, following a Poisson distribution with $\lambda = 3$, while the rest reproduced with $\lambda = 2$. The $\lambda = 3$ Poisson distribution meant that these selected individuals, on average, produced 3 offspring per generation, resulting in higher offspring production compared to the rest of the bacterial strains (Supplementary Figure 15 & 16). This represents a simple selective pressure model.

**Refined selective model**

In the fourth simulation scenario, we used a selective model similar to the third scenario, with the addition of locus-specific selection coefficients. We created a dataset following a gamma distribution to serve as the selection coefficients, with the size of the dataset corresponding to the simulated chromosome length. The scale parameter in the gamma distribution controls the magnitude of the selection coefficients: larger scale values result in fewer loci having larger selection coefficients, while the majority of loci have very low selection coefficients (close to 0). Since each locus had a specific selection coefficient, the assignment of values for each simulated strain was modified accordingly. The quantitative trait value was calculated as follows:

$$\text{score} = \sum_{i=1}^{N_{m1}} \left(m1_i \cdot \text{coefficient}_i\right) - \sum_{j=1}^{N_{m2}} \left(m2_j \cdot \text{coefficient}_j\right) \qquad \text{(Equation 4)}$$

In this formula, $N_{m1}$ and $N_{m2}$ represent the number of m1 and m2 mutations in the

simulation, respectively. This calculation differs from the previous simulations, where the trait value was simply the difference between the numbers of "m1" and "m2" mutations. We first used the previous parameters (*e.g.*, Chromosome = 1 Mb, HGT = 10 kb, and population size = 10,000) and tested three sets of experiments with a gamma-distributed dataset, where the mean value was set to 1. The scale parameters for these experiments were set to $1\times10^2$, $1\times10^4$, and $1\times10^6$ (with the final scale corresponding to the simulated chromosome length). From the top and bottom 300 individuals, 150 were selected to have increased offspring production, following a Poisson distribution with $\lambda = 3$, while the rest reproduced with $\lambda = 2$. At 10,000 generations, we obtained reliable results, and the extremity of the SNP loading values was found to be associated with SNP coefficients (Supplementary Figure 18).

To better approximate the real *K. pneumoniae* genome, we adjusted the chromosome length to 5 Mb and increased the HGT tract length, setting the length of HGT between each bacterium and others in the population to 100 kb per generation. Each locus on the chromosome was assigned a selection coefficient drawn from a gamma distribution, which included five million values, one for each locus on the chromosome, regardless of whether a mutation occurred at that locus. The scale for the gamma distribution was set to $5\times10^6$ for this simulation. Similarly, the mean value of the gamma distribution was set to 1 (shape of gamma distribution = $2\times10^{-7}$). From the top and bottom 600 individuals, 300 from each group were selected to have increased offspring production, following a Poisson distribution with $\lambda = 3$, while the remaining individuals reproduced with $\lambda = 2$ (Supplementary Figure 19).

Compared to the Selective Model, the Refined Selective Model introduces locus-specific selection coefficients, better simulating the different selective pressures at each locus, and thus more closely approximating the complexity of real-world genomes.

All the scripts can be found at https://github.com/sql647/KpBACKBONE.

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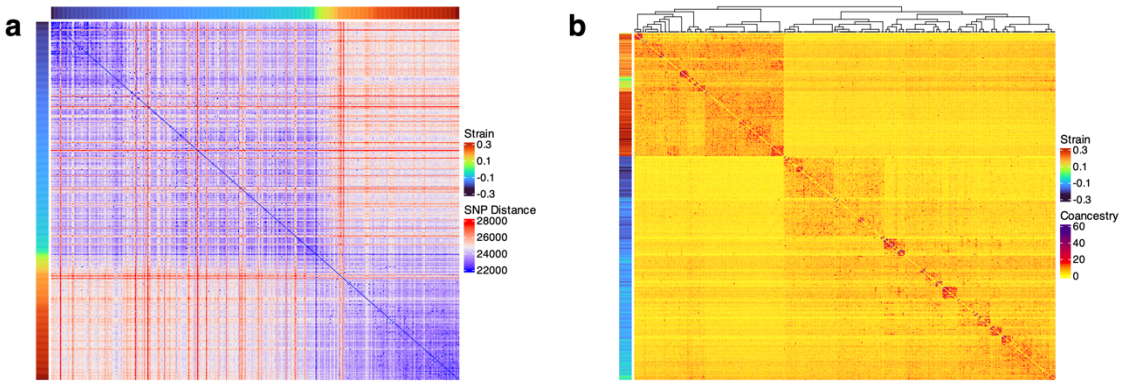
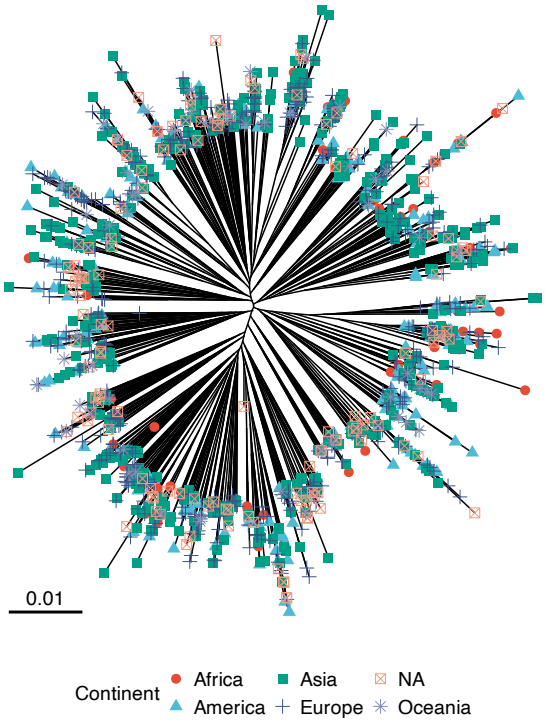
## Supplementary Figures



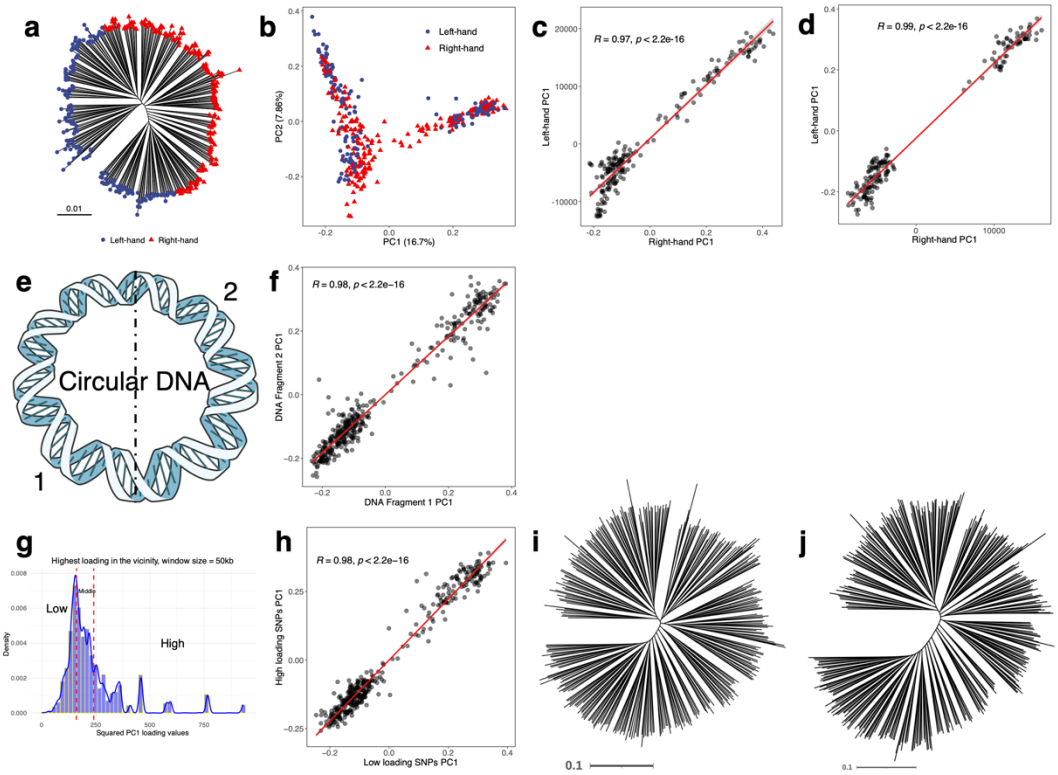

**Supplementary Figure 1.** Genomic characteristics of the non-redundant Klebsiella pneumoniae dataset.

(a) SNP distance matrix illustrating the genetic distances between strains in the non-redundant dataset, with strains ordered based on their PC1 values. The minimum SNP distance observed is 20,000 SNPs. (b) fineSTRUCTURE analysis of the non-redundant KP dataset, highlighting population structure and clustering patterns. The annotation bar indicates the PC1 values of the strains.




**Supplementary Figure 2.** Phylogenetic tree of the complete KP dataset. A maximum-likelihood phylogenetic tree constructed based on core SNPs from the full K. pneumoniae dataset, with branches

color-coded by continental origin to illustrate the global distribution of isolates.

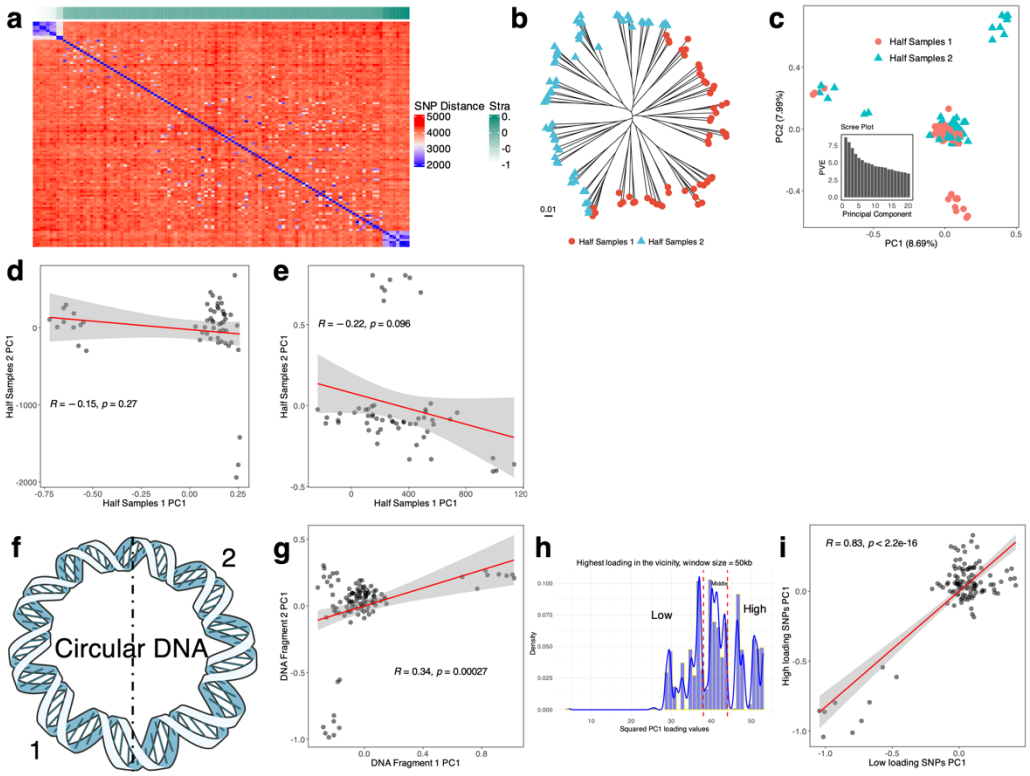



**Supplementary Figure 3.** Three types of half-matching tests for the non-redundant Klebsiella pneumoniae dataset.

(a–d) Half-Strain Matching: (a) Phylogenetic tree of the non-redundant K. pneumoniae dataset divided into two subsets based on the backbone. (b) PCA of the non-redundant K. pneumoniae dataset divided into two groups. (c) Correlation of PC1 from right-hand group mapped onto left-hand group results. (d) Correlation of PC1 from left-hand group mapped onto right-hand group results.

(e–h) Half-Segment Matching: (e) The genome is divided into two fragments: Fragment 1 (1 bp–2.6 Mb) and Fragment 2 (2.6 Mb–5.2 Mb). (h) Correlation of PC1 between the two fragments.

(g–j) Partial-Variants Matching: (h) Histogram of SNP loadings on PC1, dividing SNPs into high- and low-loading groups. (h) Correlation of PC1 between high- and low-loading SNP groups. (i) Phylogenetic tree based on low-loading SNPs. (j) Phylogenetic tree based on high-loading SNPs.



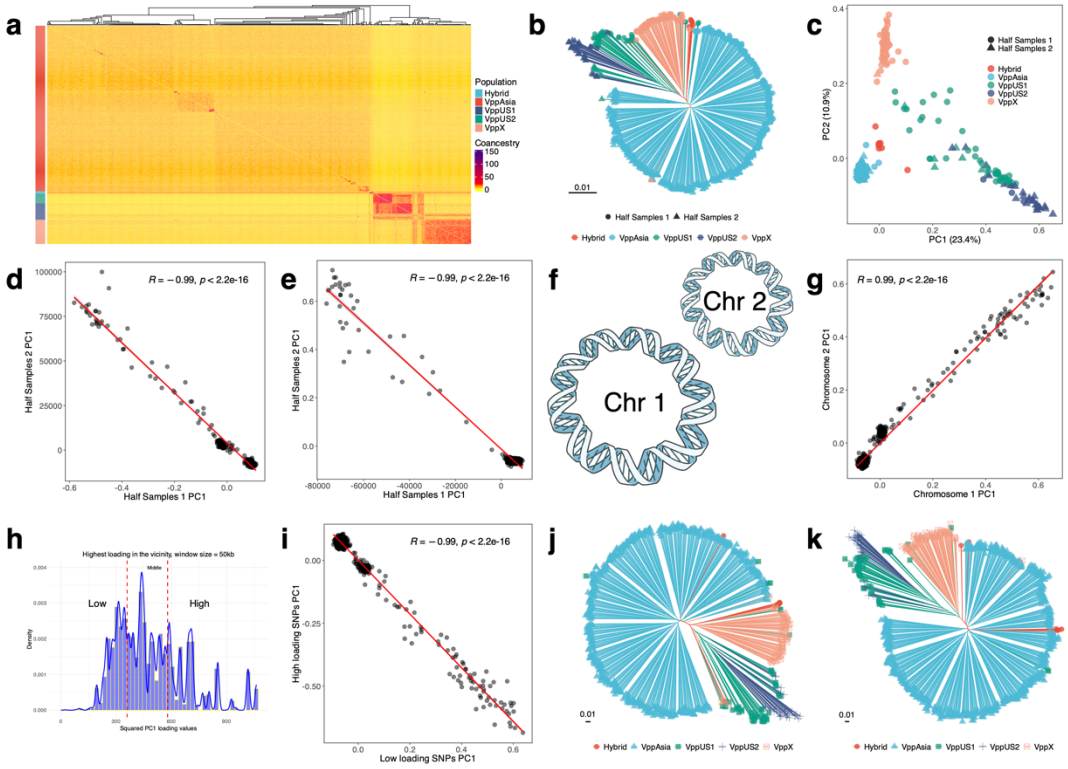

**Supplementary Figure 4.** Three types of half-matching tests for the clonal simulation.

(a) SNP distance matrix for the clonal simulation. The minimum SNP distance observed is 2,000 SNPs.

(b–e) Half-Strain Matching: (b) Phylogenetic tree of the non-redundant clonal simulation divided into two subsets. (c) PCA of the non-redundant clonal simulation divided into two groups. (d) Correlation of PC1 from group 2 mapped onto group 1 results. (e) Correlation of PC1 from group 1 mapped onto group 2 results.

(f–g) Half-Segment Matching: (f) Division of the genome into two fragments (Fragment 1 and Fragment 2). (g) Correlation of PC1 between the two fragments.

(h–i) Partial-Variants Matching: (h) Histogram of SNP loadings on PC1, dividing SNPs into high- and low-loading groups. (i) Correlation of PC1 between high- and low-loading SNP groups.



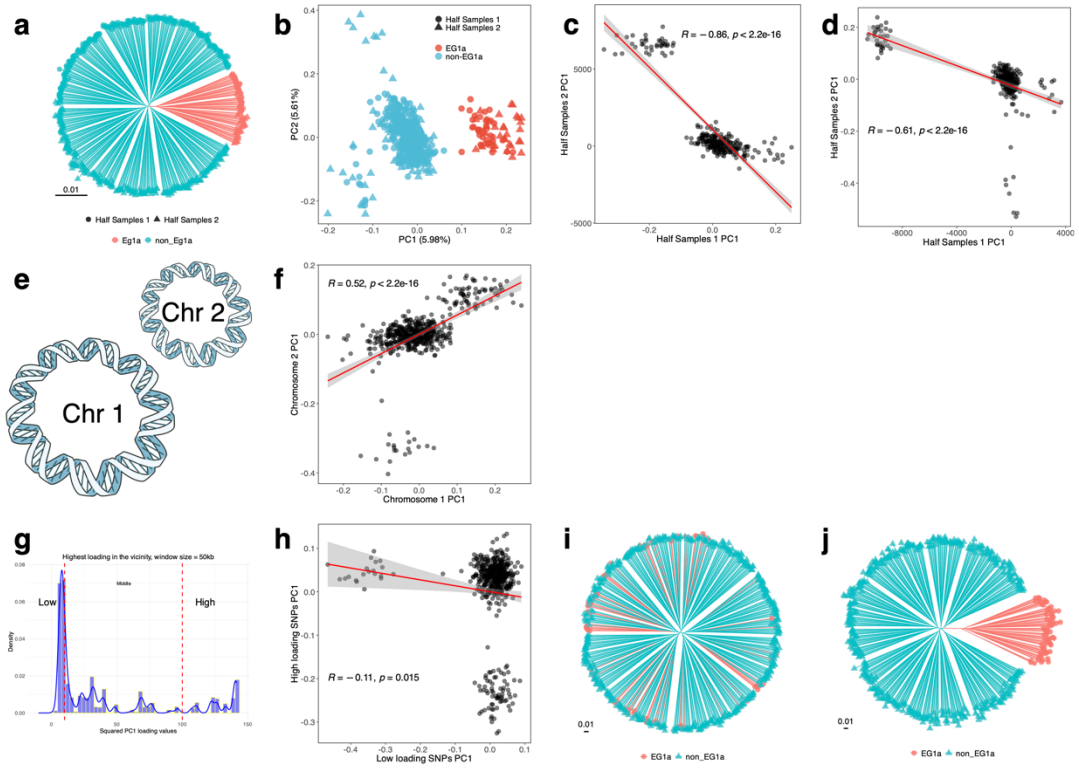

**Supplementary Figure 5.** Three types of half-matching tests for the non-redundant VP dataset.

(a) fineSTRUCTURE analysis assigning populations for the non-redundant VP dataset.

(b–e) Half-Strain Matching: (b) Phylogenetic tree of the non-redundant VP dataset divided into two subsets, with different shapes representing the groups and colors indicating different populations. (c) PCA of the non-redundant VP dataset, divided into two groups, with colors and point shapes consistent with panel A. (d) Correlation of PC1 from group 2 mapped onto group 1 results. (e) Correlation of PC1 from group 1 mapped onto group 2 results.

(f–g) Half-Segment Matching: (f) Representation of VP Chromosome 1 and Chromosome 2, with proportions reflecting their actual sizes. (g) Correlation of PC1 between the two chromosomes.

(h–k) Partial-Variants Matching: (h) Histogram of SNP loadings on PC1, dividing SNPs into high- and low-loading groups. (i) Correlation of PC1 between high- and low-loading SNP groups. (j) Phylogenetic tree based on low-loading SNPs. (k) Phylogenetic tree based on high-loading SNPs.

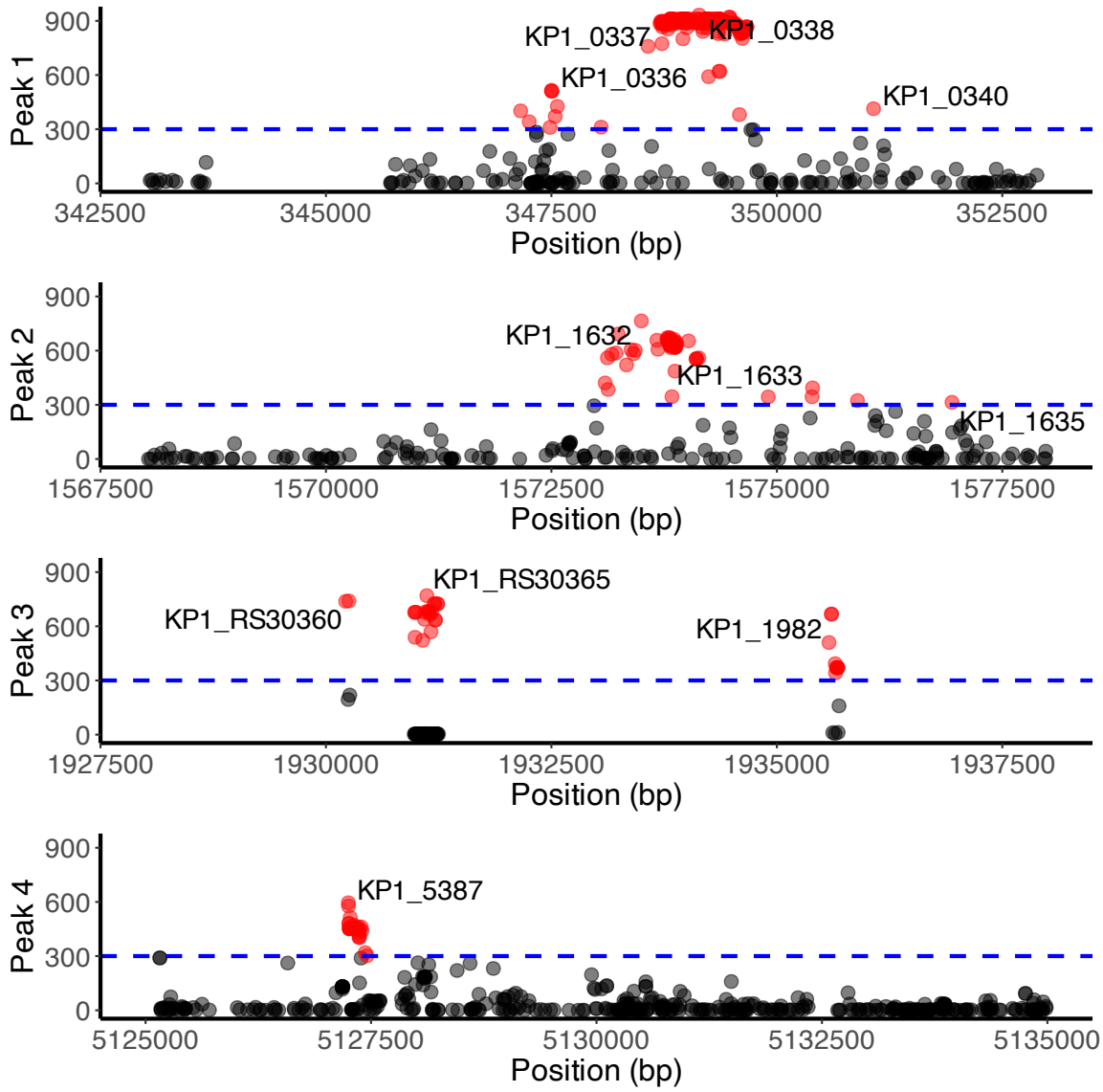



**Supplementary Figure 6.** Three types of half-matching tests for the VppAsia dataset.

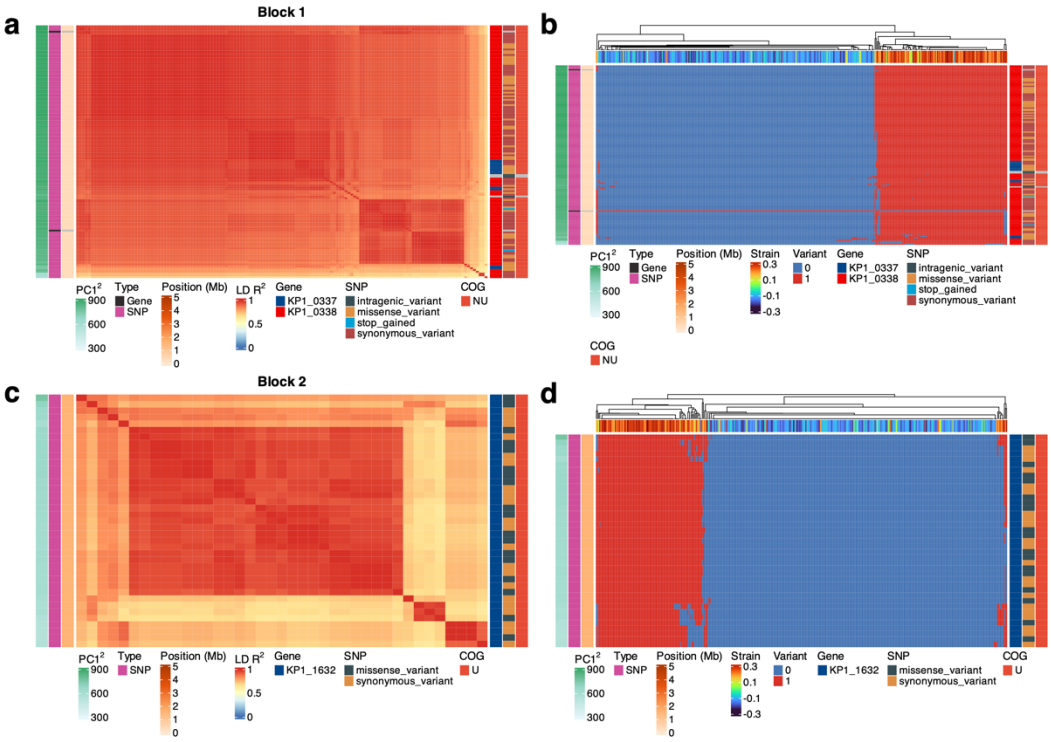
(a–d) Half-Strain Matching: (a) Phylogenetic tree of the VppAsia dataset divided into two subsets, with different shapes representing the groups and colors indicating different ecospecies. (b) PCA of the non-redundant V. parahaemolyticus Asia dataset, divided into two groups, with colors and point shapes consistent with panel A. (c) Correlation of PC1 from group 2 mapped onto group 1 results. (d) Correlation of PC1 from group 1 mapped onto group 2 results.

(e–h) Half-Segment Matching: (e) Representation of VP Chromosome 1 and Chromosome 2, with proportions reflecting their actual sizes. (h) Correlation of PC1 between the two chromosomes.

(g–j) Partial-Variants Matching: (g) Histogram of SNP loadings on PC1, dividing SNPs into high- and low-loading groups. (h) Correlation of PC1 between high- and low-loading SNP groups. (i) Phylogenetic tree based on low-loading SNPs. (j) Phylogenetic tree based on high-loading SNPs.



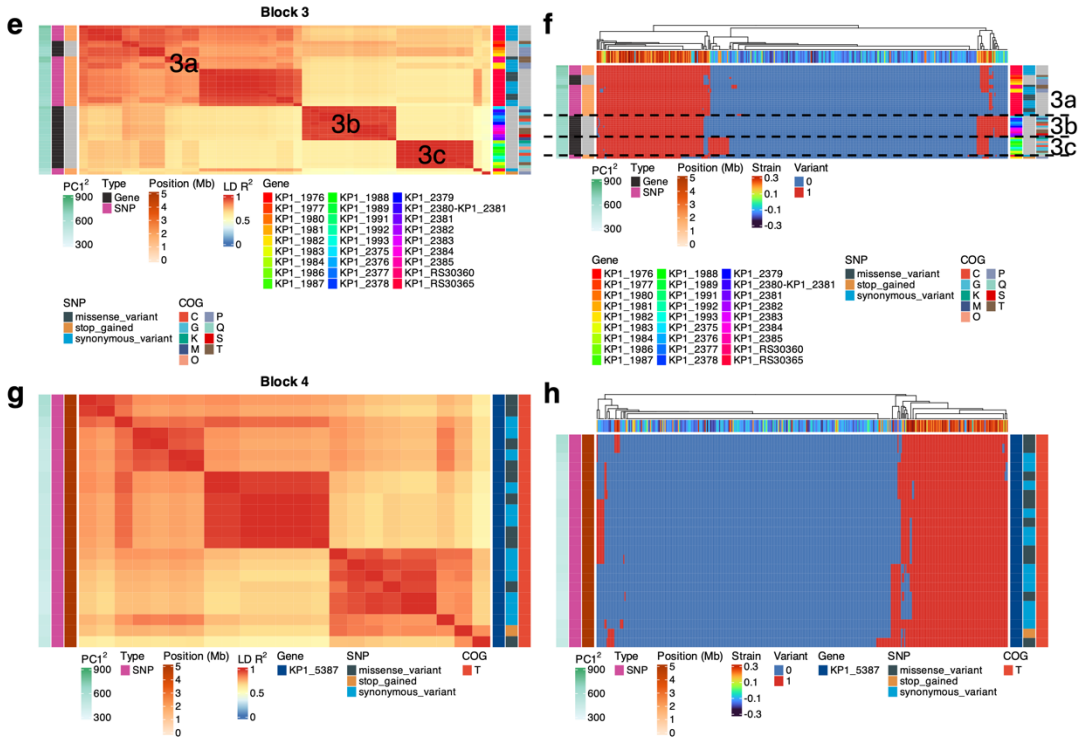

**Supplementary Figure 7.** Zoomed-in views of four high-loading SNP peaks identified across the genome.




**Supplementary Figure 8:** Overview of blocks 1–4 from LD analysis with variant status heatmaps.

Block 1 (a-b): (a) SNPs in KP1_0337, KP1_0338, and associated accessory genes form a high LD block. (b) Variant (1/0) heatmap shows clear differentiation in gene presence/absence or SNP minor/major alleles across isolates.

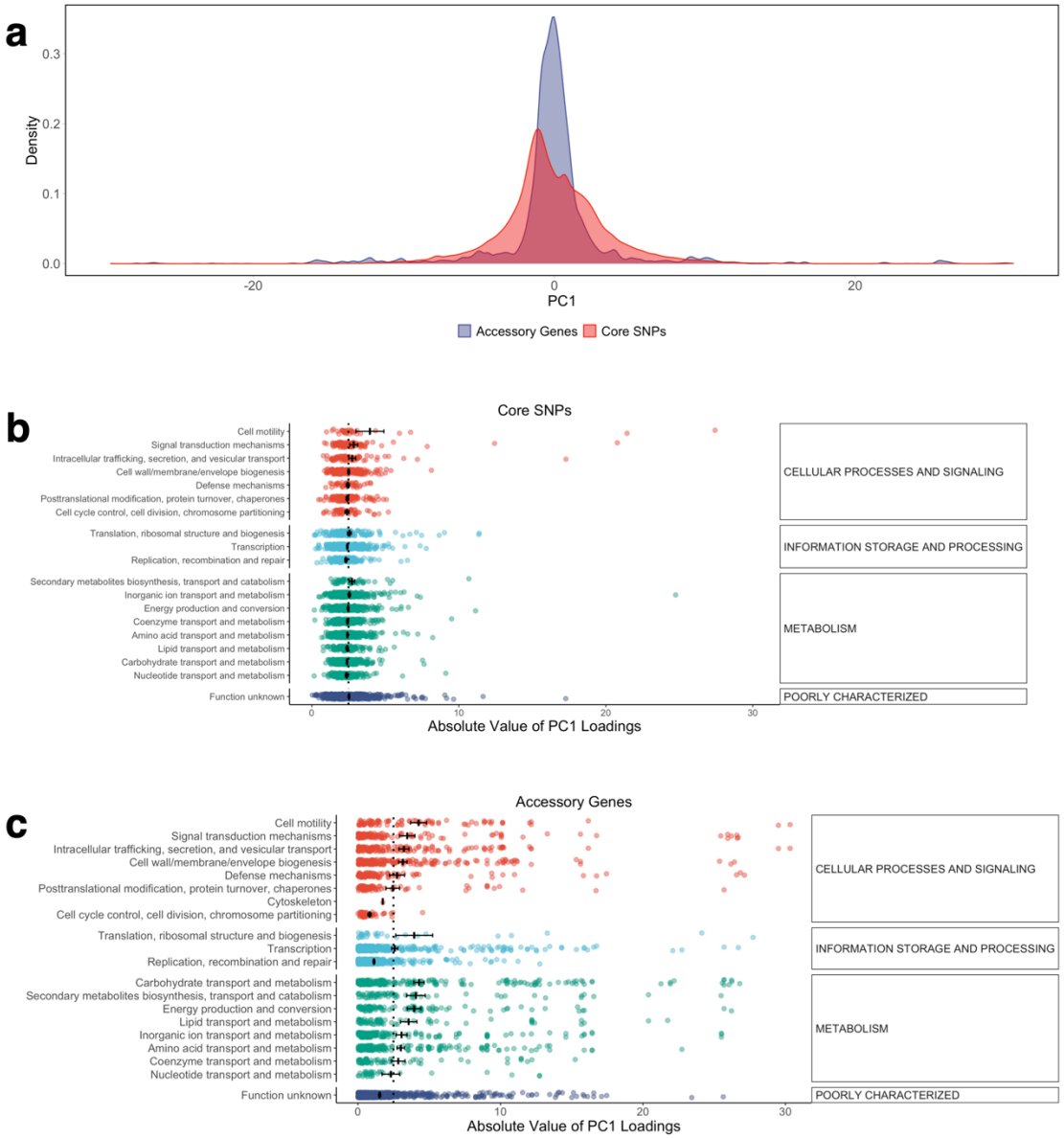
Block 2 (c-d): (c) High LD observed for SNPs in KP1_1632. (d) Variant (1/0) heatmap highlights distinct patterns of gene presence/absence or SNP minor/major alleles across isolates.




**Supplementary Figure 8:** Overview of blocks 1–4 from LD analysis with variant status heatmaps.

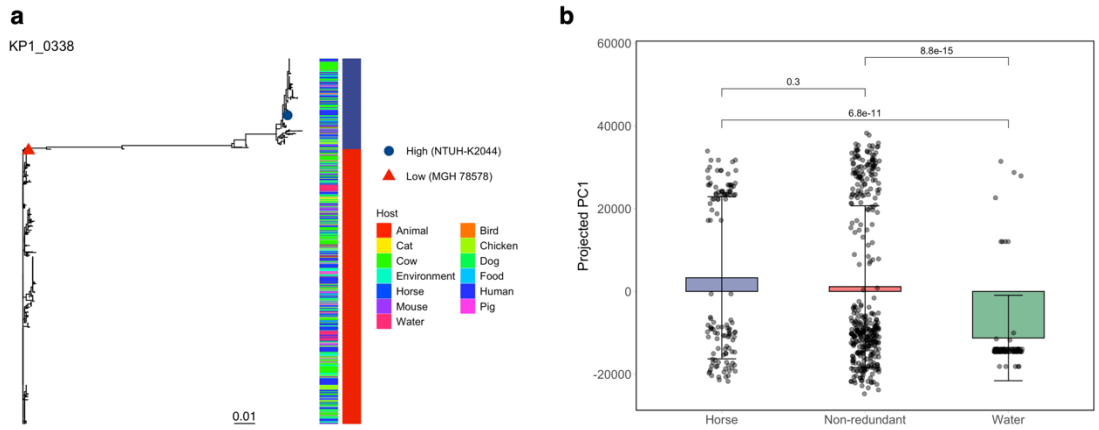
Block 3 (e-f): (e) SNPs in KP1_RS09345, KP1_RS30360, KP1_1982, and accessory genes form tightly linked clusters. (f) Variant (1/0) heatmap reveals clear differentiation in gene presence/absence or SNP minor/major alleles across isolates. Int 1 represents an intermediate case that includes Block 3b but excludes 3c, while Int 2 represents an intermediate case that includes 3c but excludes 3b.

Block 4 (g-h): (g) High LD observed for variants in KP1_5387, forming a small, highly linked cluster. (h) Variant (1/0) heatmap shows distinct variation in gene presence/absence or SNP minor/major alleles across isolates.




**Supplementary Figure 9.**

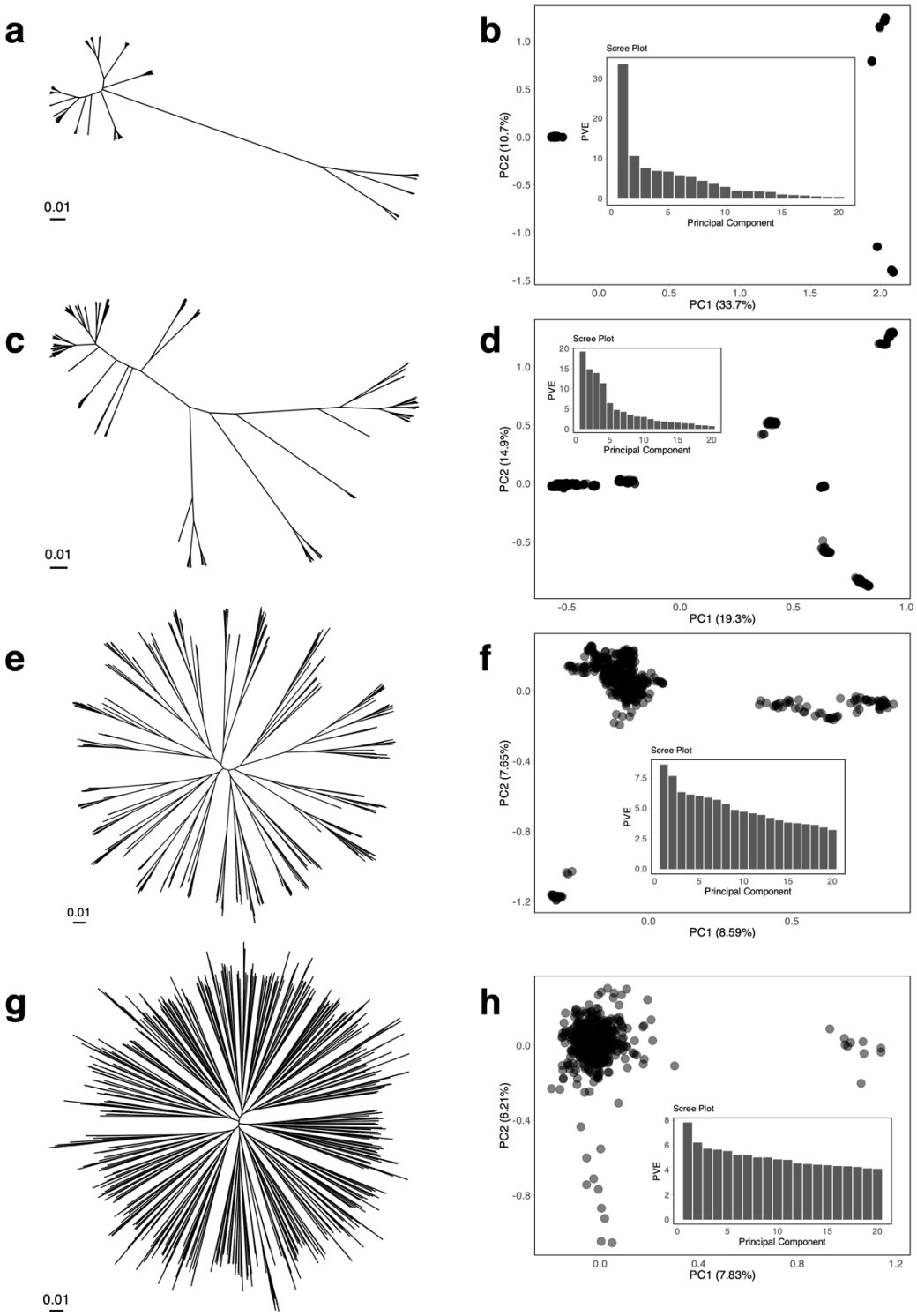
(a) Density plot of PC1 loading values for core SNPs and accessory genes. Mean absolute PC1 loadings of (b) core SNPs and (c) accessory genes grouped by COG category. Bars represent the average absolute loading within each COG category, with error bars indicating the standard error. The dashed line marks the overall mean absolute loading across all features (accessory genes or core SNPs, respectively).



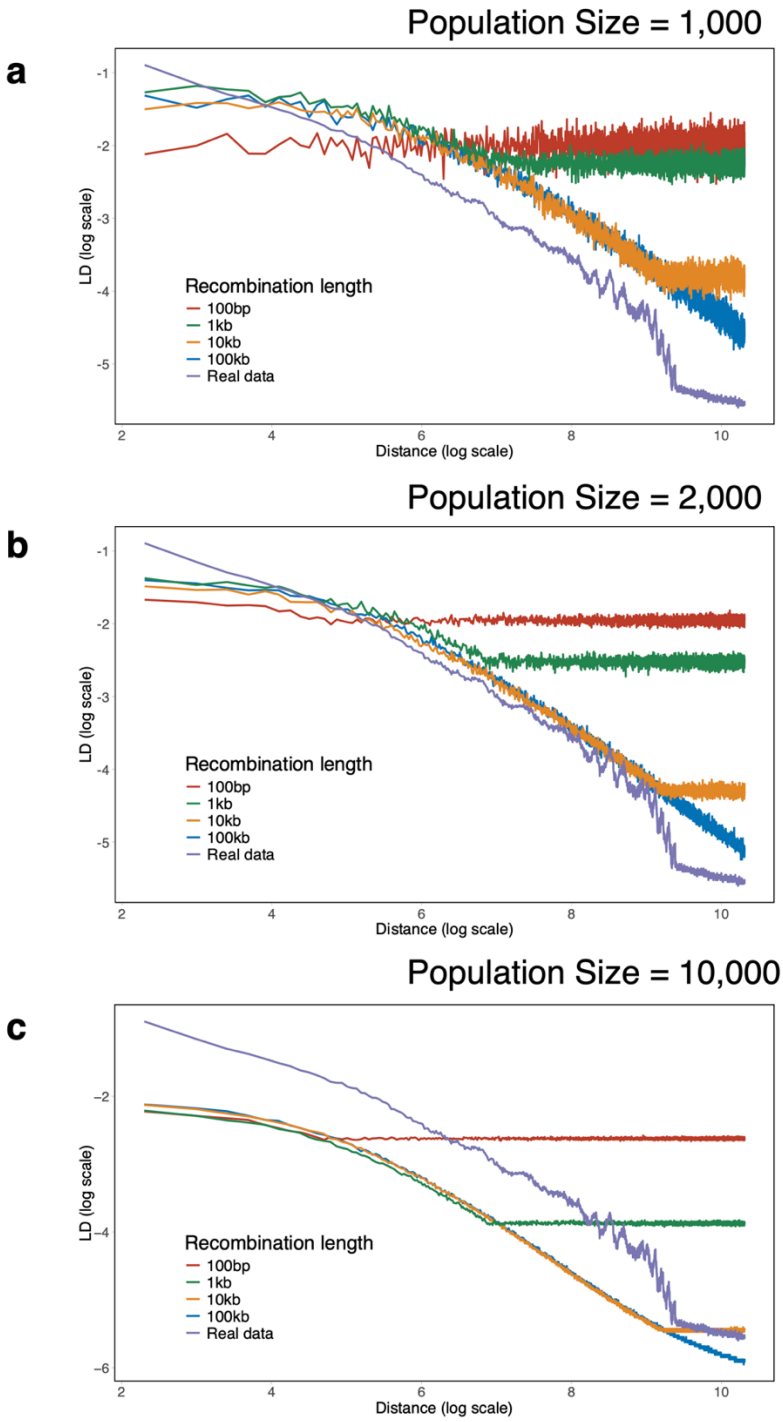

**Supplementary Figure 10.** Host origin profiles.

(a) Phylogenetic tree of the KP1_0338 gene based on sequences from the multi-host dataset. Two annotation strips indicate host origin and KP1_0338 classification (High vs. Low) based on phylogenetic clustering.

(b) Projected the multi-host dataset onto the non-redundant dataset. Each bar represents the mean PC1 value for isolates from a given host, with error bars indicating the standard deviation.



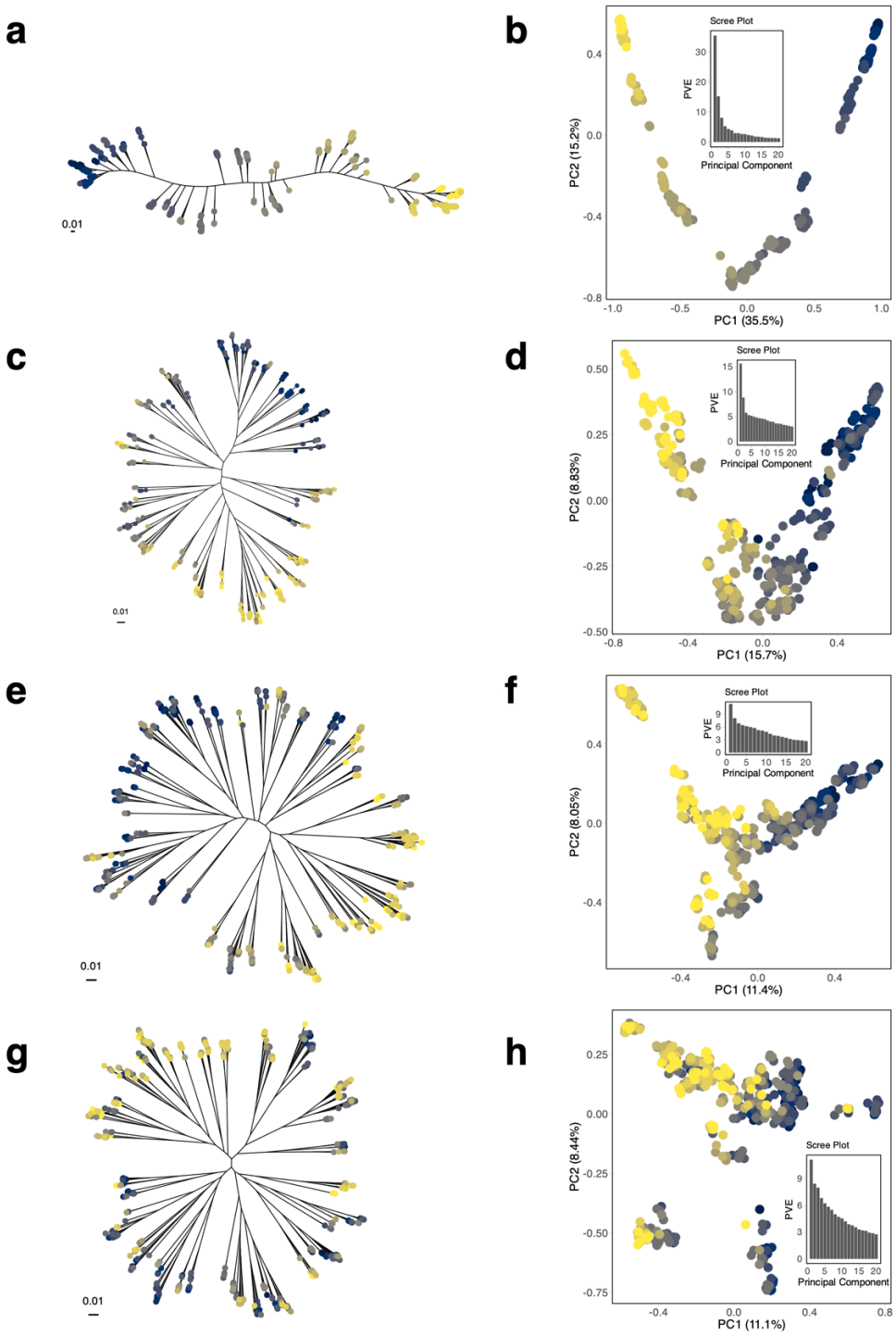

**Supplementary Figure 11.** Clonal haploid bacteria model with varying horizontal gene transfer (HGT) lengths and a population size of 2000. Phylogenetic trees, principal component analysis (PCA) plots, and scree plots for a population of 2000 individuals under different HGT lengths. Panels (a-h) show results for: (a-b) 100 bp, (c-d) 1 kb, (e-f) 10 kb, (g-h) 100 kb.




**Supplementary Figure 12.** Linkage disequilibrium (LD) decay under different horizontal gene transfer (HGT) lengths in clonal haploid bacteria models.

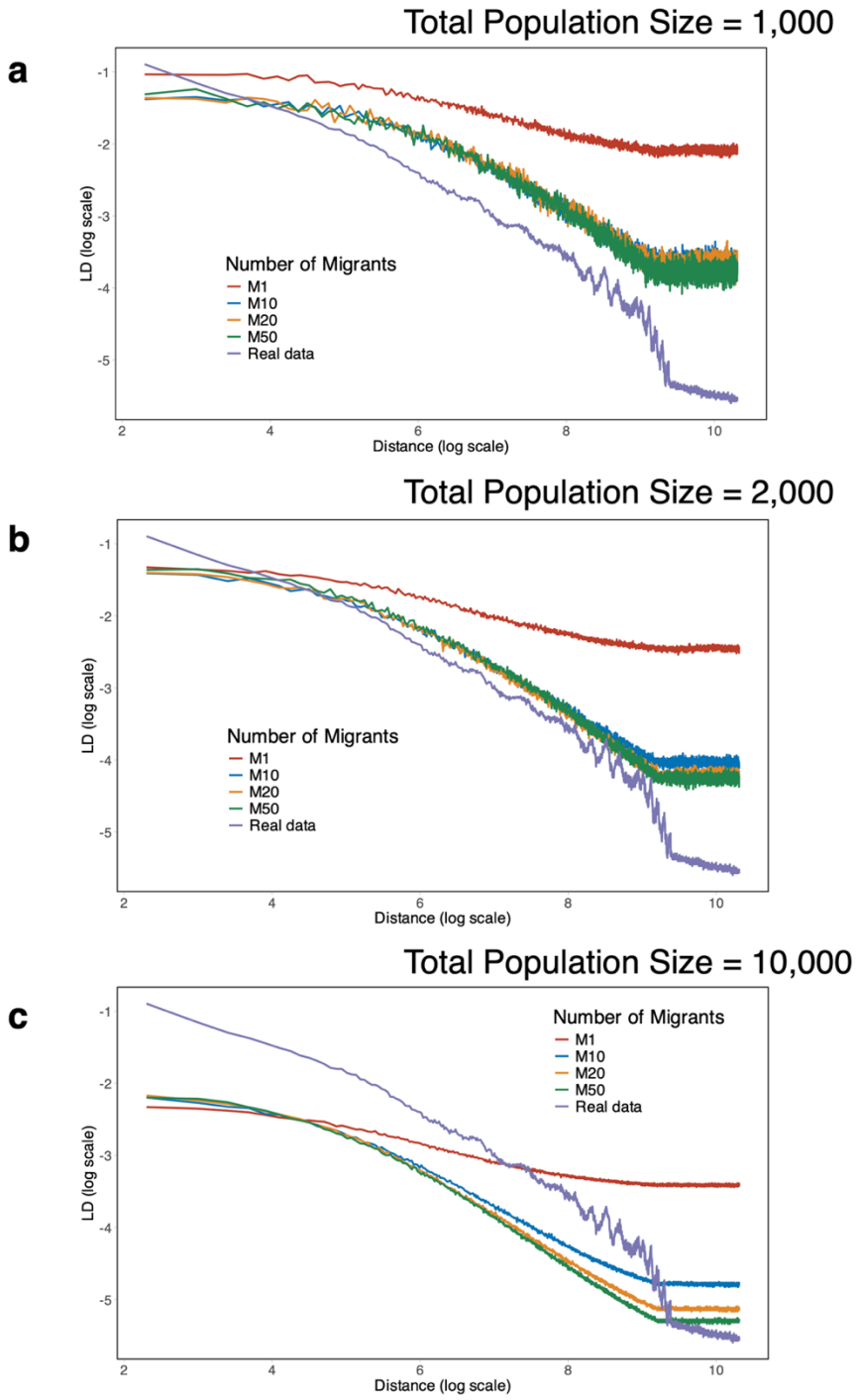
(a-c) Comparison of LD decay between different HGT lengths (100 bp, 1 kb, 10 kb, and 100 kb) and the non-redundant Klebsiella pneumoniae dataset. (a) Population size of 1,000. (b) Population size of

2,000. (c) Population size of 10,000. The x-axis represents the SNP distance (log scale), and the y-axis represents the LD value ($R^2$) on a log scale.



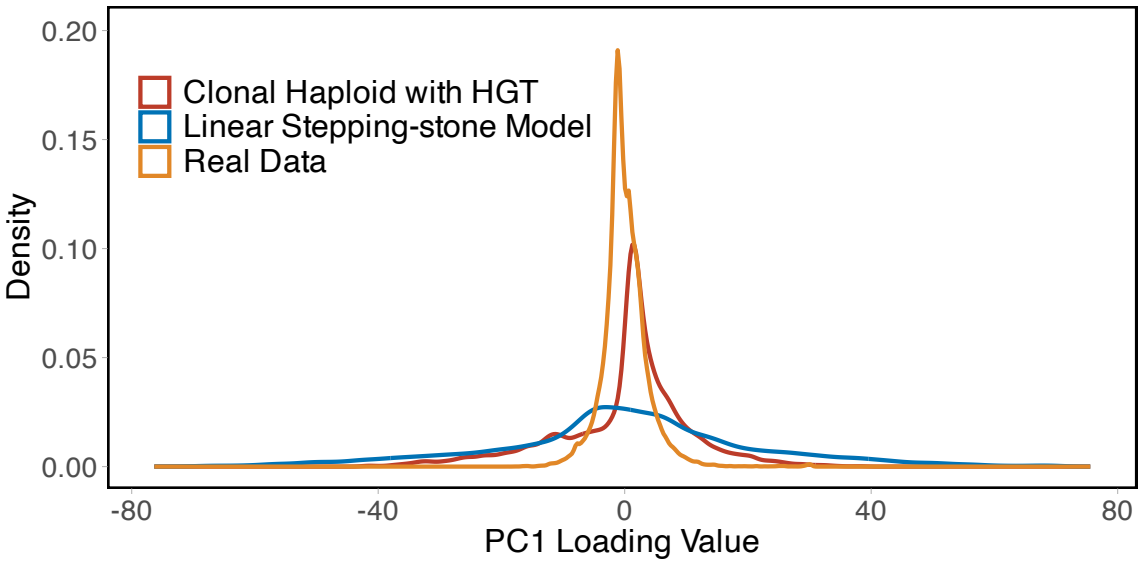

**Supplementary Figure 13.** Linear stepping-stone model with varying numbers of migrants and a population size of 2000. Phylogenetic trees, PCA plots, and scree plots for a model with HGT of 10 kb.

The population consists of 10 subpopulations (200 individuals each). Panels (a–h) show results for different numbers of migrants exchanged between neighbor subpopulations: (a-b) 1 migrant, (c-d) 10 migrants, (e-f) 20 migrants, (g-h) 50 migrants.

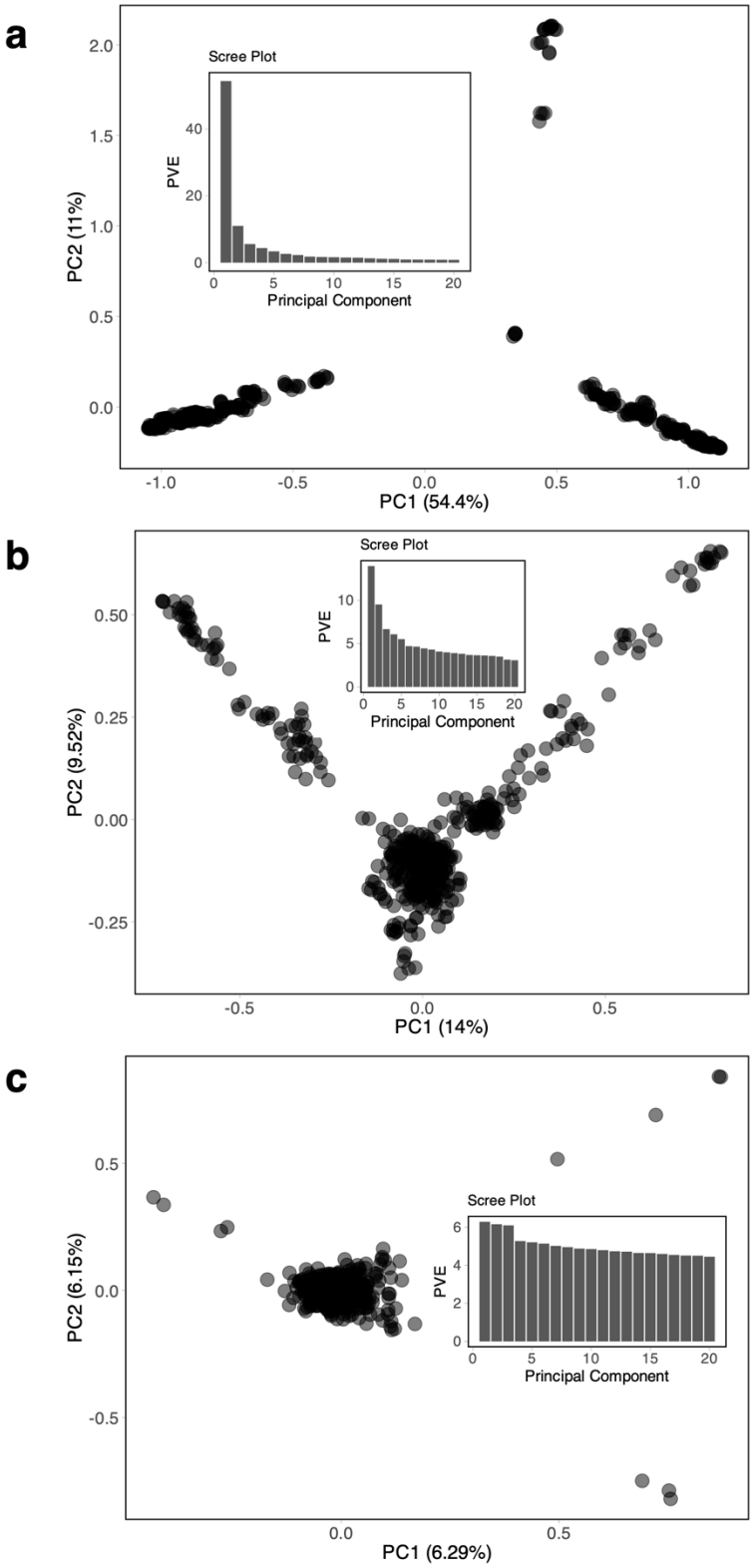



**Supplementary Figure 14.** Linkage disequilibrium (LD) decay in linear stepping-stone models

compared to the non-redundant KP data. Linkage disequilibrium (LD) decay profiles are compared between linear stepping-stone models with varying numbers of migrants (1, 10, 20, 50) and the non-redundant K. pneumoniae dataset. Panels illustrate results for different total population sizes: (a) 2000 individuals, (b) 10,000 individuals, (c) 100,000 individuals. Each model includes horizontal gene transfer (HGT) of 10 kb. The x-axis represents the SNP distance (log scale), and the y-axis represents the LD value ($R^2$) on a log scale.

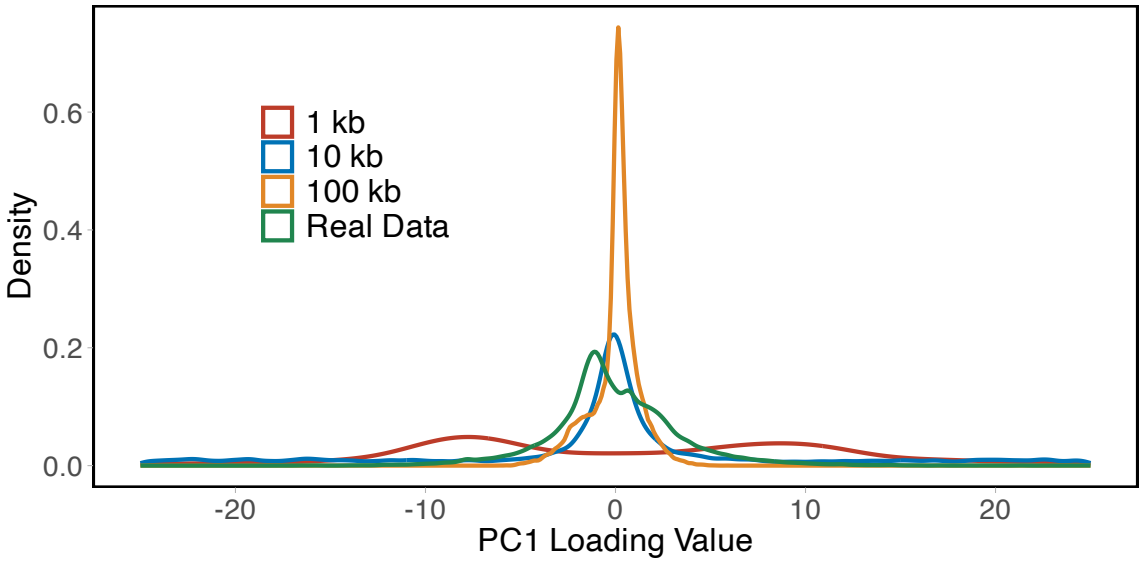



**Supplementary Figure 15.** Density plot of SNP PC1 loading values illustrating the distribution of SNP loadings for real data and two simulated models.

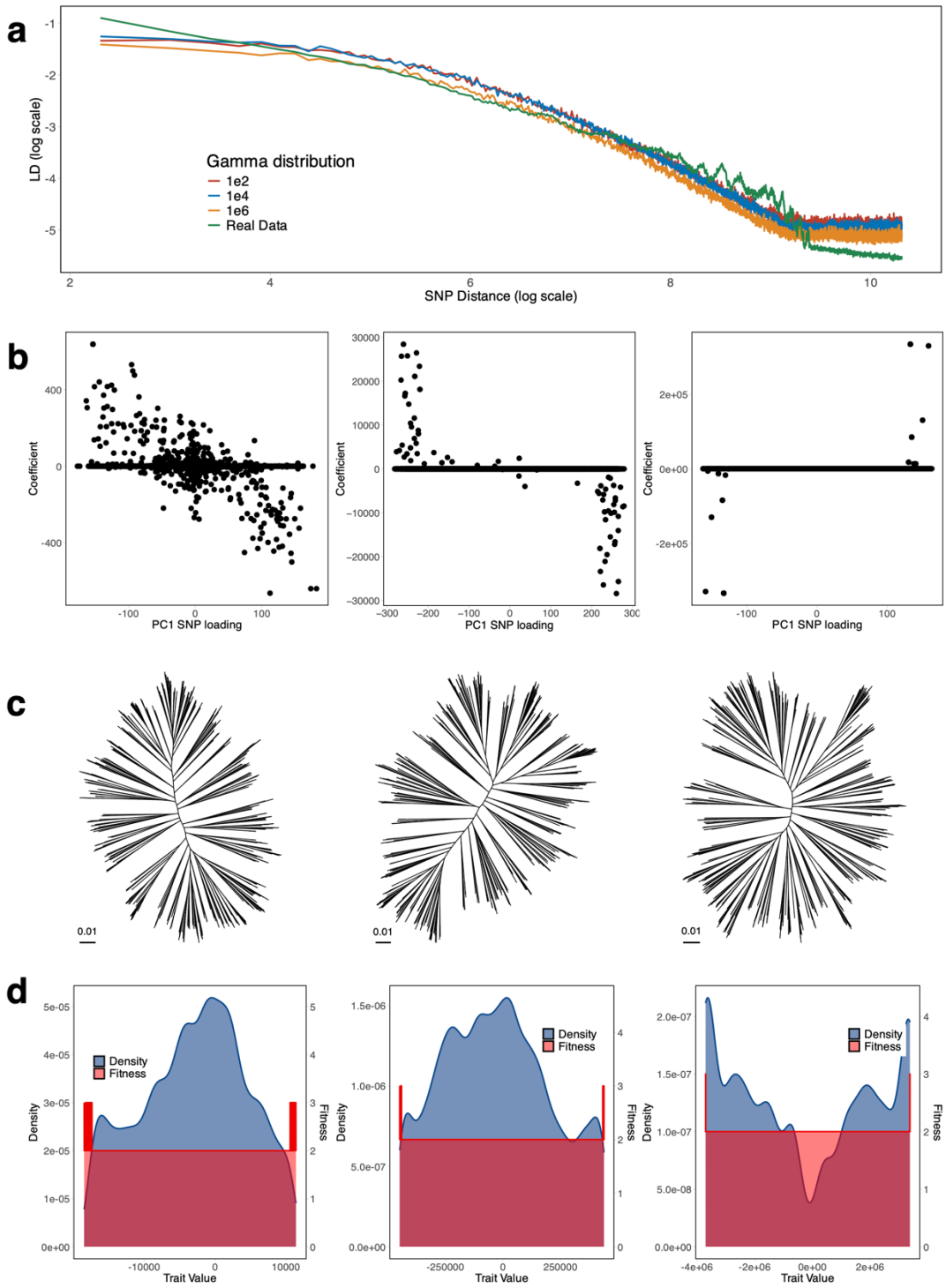



**Supplementary Figure 16.** PCA results and trait-PC1 correlation in the selection model with varying

HGT lengths. (a, b, c) PCA plots corresponding to HGT lengths of 1 kb, 10 kb, and 100 kb for 1 Mb chromosome length, respectively, in the selection model described in Figure 3.

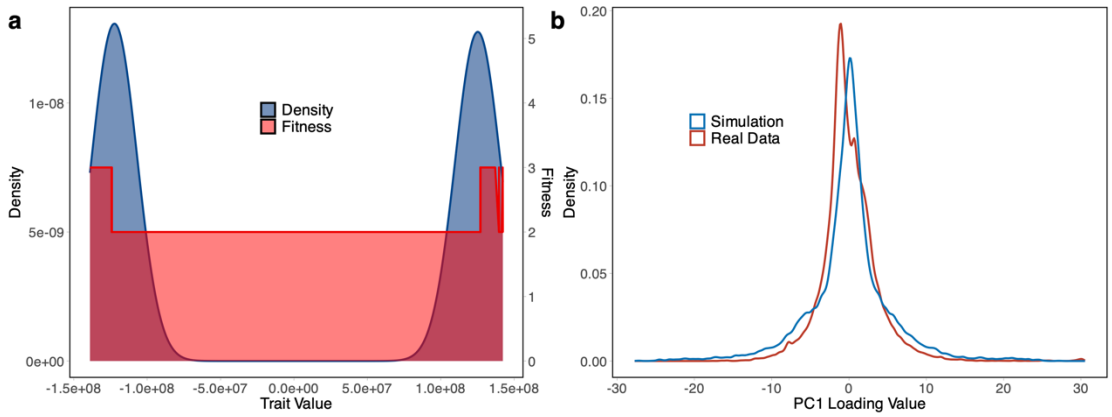



**Supplementary Figure 17.** Distributions of SNP loadings (-25~25) for horizontal gene transfer (HGT) with different lengths (1 kb, 10 kb, and 100 kb) compared to real data.




**Supplementary Figure 18.** Comparison of LD decay, phylogenetic trees, and trait-fitness relationships under the 1 Mb optimized selection model with varying gamma distribution scales for locus-specific selection coefficients. (a) LD decay curves for SNPs generated under varying gamma distribution scales ($10^2$, $10^4$, $10^6$), compared to real data. (b) Relationship between SNP coefficients

and PC1 SNP loadings for the three gamma scales. (c) Phylogenetic trees reconstructed from SNPs with gamma distribution scales of $10^2$, $10^4$, and $10^6$. (d) Distribution of trait values and fitness for SNPs under the three gamma scales, with fitness modeled using Poisson distributions for offspring numbers.




**Supplementary Figure 19.** Selection model with locus-specific selection coefficients for 5 Mb chromosome length. (a) Trait value density distributions and fitness function. (b) Distribution of PC1 SNP loadings for the 5 Mb simulation compared to the real data.