Robust analysis of prokaryotic pangenome gene gain and loss rates with Panstripe

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Horizontal gene transfer (HGT) plays a critical role in the evolution and diversification of many microbial species. The resulting dynamics of gene gain and loss can have important implications for the development of antibiotic resistance and the design of vaccine and drug interventions. Methods for the analysis of gene presence/absence patterns typically do not account for errors introduced in the automated annotation and clustering of gene sequences. In particular, methods adapted from ecological studies, including the pangenome gene accumulation curve, can be misleading as they may reflect the underlying diversity in the temporal sampling of genomes rather than a difference in the dynamics of HGT. Here, we introduce Panstripe, a method based on Generalised Linear Regression that is robust to population structure, sampling bias and errors in the predicted presence/absence of genes. We demonstrate using simulations that Panstripe can effectively identify differences in the rate and number of genes involved in HGT events, and illustrate its capability by analysing a number of diverse bacterial genome datasets representing major human pathogens. Panstripe is freely available as an R package at `https://github.com/gtonkinhill/panstripe`.

pangenome | microbial genomics | horizontal gene transfer
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Introduction

Genetic variation within microbial populations is shaped by both the accumulation of variation from point mutations as well as from the acquisition and loss of genetic material through Horizontal Gene Transfer (HGT). HGT can occur via the uptake of DNA from the environment, with the help of Mobile Genetic Elements (MGEs) (phages, integrative conjugative elements and plasmids) or from direct contact between bacterial cells (1). Genes are also frequently duplicated and lost vertically upon cell division (2). The influence of these sources of variation varies dramatically by species. Clonal species such as *Mycobacterium tuberculosis* typically accumulate variation nearly entirely through point mutations while naturally transformable species such as *Streptococcus pneumoniae* and *Neisseria meningitidis* have very high rates of homologous recombination (3). In other species such as *Salmonella enterica* horizontal exchange is generally restricted to the movement of MGEs (4). While HGT does not always have an impact on a microbe's fitness, they can lead to critical phenotypic changes such as the acquisition of antimicrobial resistance, virulence factors and vaccine escape (5, 6).

A common approach to analysing horizontal exchange in microbial genomics is to group homologous gene sequences into orthologous and paralogous gene clusters. The union of these clusters within a particular species or group is commonly referred to as the pangenome (7). Genes are often further classified into either the 'core' genome which are found in almost all members of the group and the 'accessory' genome which are only found in a subset of genomes. Species with a limited accessory genome such that all genes are likely to have already been observed are often described as 'closed' while species with a diverse accessory genome such that each additional genome contributes novel genes are described as 'open'.

A number of tools have been developed to infer a pangenome given a collection of annotated genomes (8–13). A common output of these approaches is a binary gene presence/absence matrix where genomes are represented by rows and orthologous gene clusters by columns. After generating a gene presence/absence matrix, researchers are often interested in comparing the size of pangenomes between datasets, determining the rate of horizontal gene exchange as well as identifying whether a pangenome is 'open' or 'closed'.

To answer these questions, researchers frequently generate a gene accumulation curve as is often done in ecological studies of species diversity (7, 14). Here, the number of unique gene clusters identified is plotted against the number of genomes. The order in which the genomes are considered is usually permuted randomly to account for the variation that this order could have on the resulting plot. In some cases, a power law such as Heaps' or Zipf's law is fit to this curve to give a parameter estimate of the diminishing number of new genes found with each additional genome and to determine whether the pangenome is open or closed (15).

A neglected problem with this approach is that it fails to account for the underlying diversity of the set of sampled genomes. For example, a set of genomes taken from within an outbreak is likely to involve far fewer gene exchange events than a diverse sample from a species with thousands of years of evolution separating isolates. Phylogenetic methods that account for the underlying diversity of the sample help to address this issue. These often make use of a phylogeny constructed from the genetic diversity present in genes found in all the genomes of a sample and include methods based on maximum parsimony (16), maximum likelihood (17–19) and Bayesian phylogenetics (20). Two notable models that use

this approach are the Infinitely Many Genes (IMG) model and the Finitely Many Genes model (FMG) (21–24). The IMG model assumes an infinite pool of genes and that a particular gene can only be gained once while the FMG model assumes that genes belong to a finite pool and that multiple gene gain and loss events of the same gene can occur. Many models also collapse paralogous clusters into gene families prior to inference of gene gain and loss rates (16, 17, 19).

A significant limitation of these approaches is that they generally assume that there is no error in the inferred pangenome presence/absence matrix. We and others have shown that gene annotation errors and the complexities of clustering genes into orthologous families can introduce substantial numbers of erroneous gene clusters (12, 13, 19, 25). While a subset of models do account for errors in the predicted presence/absence of genes, these have mostly been optimised for the analysis of eukaryotes and focus on a small number of gene families involving multiple genes (19). Most models also make the simplifying assumption that genes are gained or lost individually which can significantly bias estimates of the rate of gene exchange, particularly when the exchange of MGEs is frequent.

To address these limitations we have developed Panstripe, an approach that compares the rates of core and accessory genome evolution to account for both population structure and errors in the pangenome gene presence/absence matrix. We demonstrate using extensive simulations, and by analysing a diverse range of bacterial genome datasets that Panstripe is: (i) able to effectively compare the gene exchange rates between pangenomes, (ii) can be used to determine if there is a temporal signal in the accessory genome, and (iii) can identify if the size of gene exchange events differs between pangenomes.

Results

Overview. Panstripe accepts a phylogeny produced using standard pipelines and a corresponding gene presence/absence matrix as produced by most pangenome inference tools (10, 12, 26). The length of each branch in the phylogeny is then compared to the number of gene gain and loss events inferred to have occurred on that branch using a Generalised Linear Model (GLM) (Figure 1). The ancestral gene gain and loss events are inferred for each branch of the phylogeny using either a maximum parsimony or optionally a maximum likelihood method (27–29). This is a similar approach to the root-to-tip regression that is commonly used to test for temporal signal in phylogenies (30). However, in contrast to TempEst, the regression is not performed on the root-to-tip distance but rather on the individual branch lengths. This avoids the problematic dependence structure which makes the root-to-tip regression not suitable for statistical hypothesis testing (30, 31).

Panstripe assumes that the number of gene gain and loss events on each branch are distributed according to a Compound Poisson distribution. This allows for multiple genes to be gained and lost in a single event (see Methods). Errors in the gene presence/absence matrix introduced at either the sequencing, assembly, annotation or pangenome clustering stage are unlikely to correlate with the core genome phylogeny. Consequently, ancestral state reconstruction of these errors will place them at the tips of the phylogeny. By including a binary covariate which indicates whether or not a branch is located at the tip of the phylogeny we can control for the presence of errors in the pangenome. Without careful consideration of the individual gene sequences it is not possible to accurately distinguish errors from very rare genes that are observed once. As a result, Panstripe groups the signal from both these sources into a single term.

The GLM framework used by Panstripe can also be used to compare the rates of HGT between pangenomes or to identify associations with additional covariates of interest. This relies on the given phylogenies having the same scale which can be achieved by using timescaled trees. Alternatively, SNP scaled phylogenies can be used if differences between the ratio of core to accessory genome variation is of interest. To test whether the number of genes involved in each recombination event is significantly different between two pangenomes, Panstripe allows different models to be fit separately to the mean and dispersion structure of the GLM (32).

The Panstripe package provides a number of plotting functions including an alternative to the popular pangenome accumulation curve that controls for both errors and differences in the core genome diversity of the underlying isolates. The package is written in R and is available freely under an MIT licence from https://github.com/gtonkinhill/panstripe.

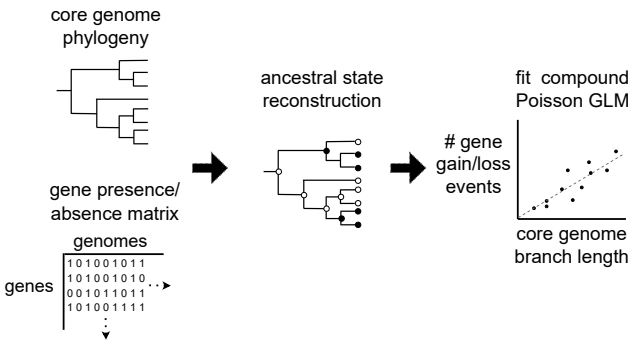

Fig. 1. A schematic of the Panstripe algorithm. A binary gene presence/absence matrix and a core genome phylogeny are taken as input. The ancestral states of each gene is then determined before fitting a compound Poisson GLM to compare the core branch length with the number of gene gain and loss events on each branch. Additional terms are included to account for the depth of a branch and whether or not it occurs at the tips of the phylogeny.

Panstripe is robust to errors in pangenome clustering. To assess the robustness of the Panstripe algorithm to the choice of pangenome clustering method, we considered the pangenome analysis of a large outbreak of highly clonal *Mycobacterium tuberculosis* (Mtb) in London spanning 14 years (33). This collection of genomes has been previously analysed using both the Roary and Panaroo pangenome clustering pipelines (12). Due to the very low mutation rate and highly clonal nature of the outbreak we would expect there to be no pangenome variation in this dataset making it a useful control for assessing whether pangenome inference tools are

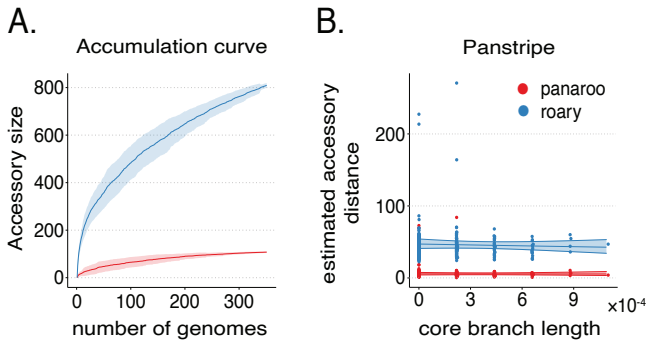

Fig. 2. A.) Pangenome accumulation curves after running both Roary (blue) and Panaroo (red) on 351 Mtb genomes from an outbreak in London. The ribbon indicates the variation in the curve found by permuting the genome order 100 times. **B.)** Dot-plot of the core branch length versus the number of accessory genes for the same set of Mtb genomes overlaid with the Panstripe model fit. The limited number of unique branch lengths is due to the very low core genome sequence diversity. Here, the ribbon indicates the 95% confidence interval of the Panstripe model fit.

able to account for errors.

Figure 2A presents the pangenome accumulation curves of the resulting gene presence/absence matrices output by the Roary and Panaroo pipelines. The very large difference in the two curves demonstrates that the accumulation curve method is highly sensitive to the different error rates of the two pipelines. We have previously shown that Panaroo is able to significantly reduce the number of errors when generating a pangenome clustering as observed in Figure 2A. However, Panaroo still estimates a small amount of accessory variation in this clonal Mtb dataset.

Figure 2B indicates that the Panstripe algorithm is able to accurately account for the differences in error rates of the two pipelines. By plotting the core branch lengths against the number of gene gain and loss events it becomes clear that the large number of accessory genes identified by the Roary pipeline do not correlate with the core phylogeny and thus are likely to be erroneous. In both cases Panstripe correctly finds no significant association between the core and accessory variation ($p=0.461$ and $p=0.570$ respectively) which is consistent with the closed pangenome of Mtb.

Panstripe can also be used to compare the differences in the rate of gene exchange events associated with the tips of the phylogeny. Here, it correctly identifies a highly significant difference indicating the elevated rate of errors in the Roary gene presence/absence matrix ($p<0.001$).

Panstripe outperforms alternative methods on simulated data. Simulations indicate that Panstripe is significantly more robust to the impacts of errors and population structure than alternative methods including those based on information theory (15, 34) and phylogenetically informed approaches (22–24) (Figure 3). To simulate pangenome dynamics, we first simulated core genome phylogenies before simulating the number of size of gene gain and loss events along each branch (see Methods). Errors in the pangenome matrix were generated by randomly adding or removing single entries in the matrix. As many of the tools considered take different approaches and estimate different parameters we chose the main parameter of each tool and tested whether the estimate given for this parameter varied significantly at different error rates (a full list of the parameters chosen is given in Supplementary Table 1). There is no clear parameter to select when using an accumulation curve so we report the median number of accessory genes found in 20 genomes across 100 permutations.

Figure 3A indicates that Panstripe was the only tool that consistently provided similar parameter estimates even at high error rates. The median number of accessory genes for the pangenome accumulation curve varied considerably, indicating it was not robust to errors. Similarly, the Heaps' power law which is frequently used to determine if a pangenome is 'open' or 'closed' was very sensitive to errors in the gene presence/absence matrix. To compare each method's ability to account for underlying phylogenetic structure, we simulated five large phylogenies and accompanying gene presence/absence matrices. We then selected a small sub-clade (≥ 30 genomes) within each simulation to create a smaller, more closely related dataset. Here, the simulation parameters of the large dataset and the sub-clade are the same and thus each method should estimate similar parameter values. Figure 3B indicates that the phylogenetically informed methods (Panstripe, panicmage, Zamani-Dahaj, Collins) were able to accurately account for the underlying population structure. In contrast, both the pangenome accumulation curve and fitting a Heaps' power law gave substantially different estimates for the small data subset. The high sensitivity of these methods to population structure suggests that they can provide misleading signals of the dynamics of the accessory genome.

To verify that Panstripe is able to identify differences in pangenome dynamics we created three separate simulation studies where the rate of gene gain and loss, error rate and the number of genes involved in each recombination event was varied. Supplementary Figures 1 indicates that Panstripe is able to accurately identify differences in the gene exchange rate despite high error rates in the simulated gene presence/absence matrix. Similarly, Supplementary Figure 2 & 3 show that Panstripe can accurately differentiate between both the enrichment of gene gain and loss events at the tips of a phylogeny and the average size of gene gain and loss events between pangenomes.

Panstripe accurately identifies within and between species differences in gene gain and loss rates. To compare the estimates of the Panstripe algorithm on species that are known to have a diverse accessory genome, we considered two previously described datasets. The first included 315 *Enterococcus faecalis* genomes from three major hospital associated clades sampled in the Netherlands, Spain and Portugal which were clustered using the PopPUNK algorithm (35, 36). *E. faecalis* is both a commensal and nosocomial pathogen and is able to successfully inhabit a wide range of host niches. The ecologically generalist lifestyle of the microbe is facilitated by a diverse accessory genome with little association between specific niches and particular accessory genes (37, 38). Instead, adaptation to the hospital associated niche is thought to be due to selection for survivability in a broader set of niches (35). We generated pangenome curves

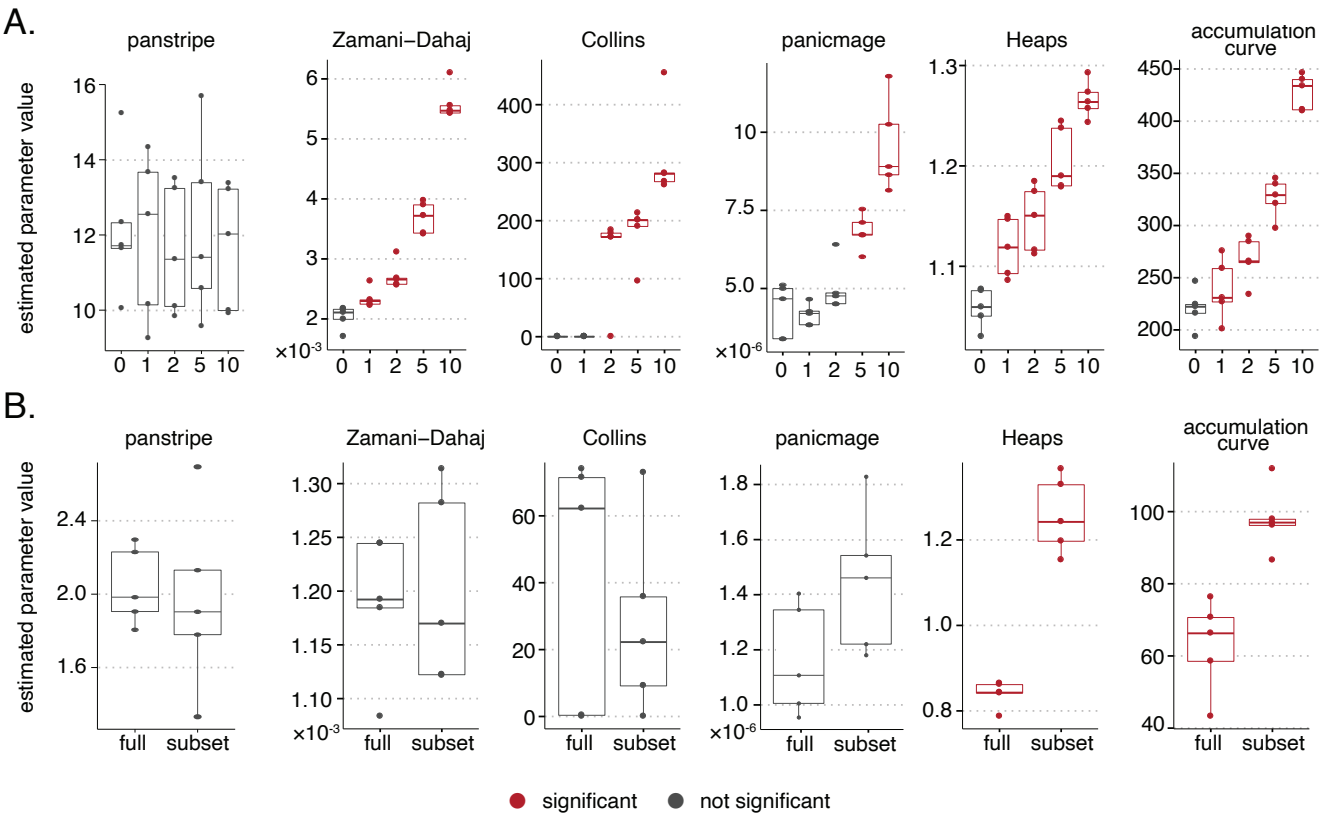

Fig. 3. A.) the estimated value of a representative parameter for each pangenome inference method across five different simulated error rates (0,1,2,5,10) with the same gene gain and loss rate. Five replicates were obtained for each set of parameter values. Estimated values that differed significantly from those with no error present according to a two-sided t-test are indicated in red. **B.)** as in A. but instead of varying the error rate each inference method was also run on a sub-clade of at least 30 genomes from the larger simulation.

and ran the Panstripe algorithm on three of the major hospital associated *E. faecalis* clades (Figure 4A,B).

Although the PopPUNK cluster (pp18) clade (including sequence types ST159 and ST525) appeared to have a more diverse accessory genome according to the pangenome accumulation curve, the Panstripe algorithm revealed that this was likely to be driven by differences in the underlying core genome diversity of the genomes being sampled. Instead, the pp6 clade (mainly of ST28) had a higher rate of gene exchange when compared to both the pp2 (mainly of ST6) ($p<0.003$) and pp18 ($p=0.017$) clades. The pp6 clade is the most recent hospital associated clade to have emerged in this dataset and thus the elevated rate of gene exchange could be a result of the additional selection pressures acting within the hospital environment which may drive the acquisition of antimicrobial resistance plasmids (35). Unlike the association with the core branch length, we did not find evidence for a difference in the size of gene exchange events and found only a small difference in the number of genes associated with the tips of the pp2 and pp6 phylogenies.

The second dataset consisted of four subclades (A, B, C1, C2) of the globally disseminated ST131 clone from a longitudinal study of *Escherichia coli* isolates from the Norwegian surveillance on resistant microbes (NORM) program (39). For brevity, these are subsequently referred to as 'clades' in the text. Similar to our analysis of *E. faecalis* the estimated pangenome accumulation curve for these clades gave strikingly different results to that produced by Panstripe. According to the pangenome accumulation curve, Clade B exhibited the largest accessory genome diversity. However, this is likely due to the increased age of Clade B as its expansion in the Norwegian population occurred nearly a decade earlier than the other clades (39). Thus, the pangenome accumulation curve is again simply reflecting the underlying population structure of the dataset rather than any difference in the accessory genome dynamics of the clades. Instead, Panstripe predicts an increased rate of gene gain and loss for the C1 and C2 clades which expanded more recently and have a higher prevalence of drug resistance loci. The emergence of C1 and C2 clades have been shaped by the acquisition of specific F-plasmids involving a series of plasmid gene gain and losses and the exchange of gene modules mediated by the insertion sequence IS26 (40). The increased rate of gain and loss in these two clades is consistent with them acquiring and exchanging multi-drug resistant (MDR) plasmids more easily than Clade A which has also undergone a recent expansion but is generally more susceptible to different classes of antibiotics (39).

As we have dated phylogenies for both the *E. faecalis* and *E. coli* datasets it is possible to use Panstripe to compare the rates of gene gain and loss between the two species. We found that the *E. coli* clades generally had significantly higher rates of gene gain and loss than the *E. faecalis* clades. This is in agreement with the more generalistic lifestyle of *E.*

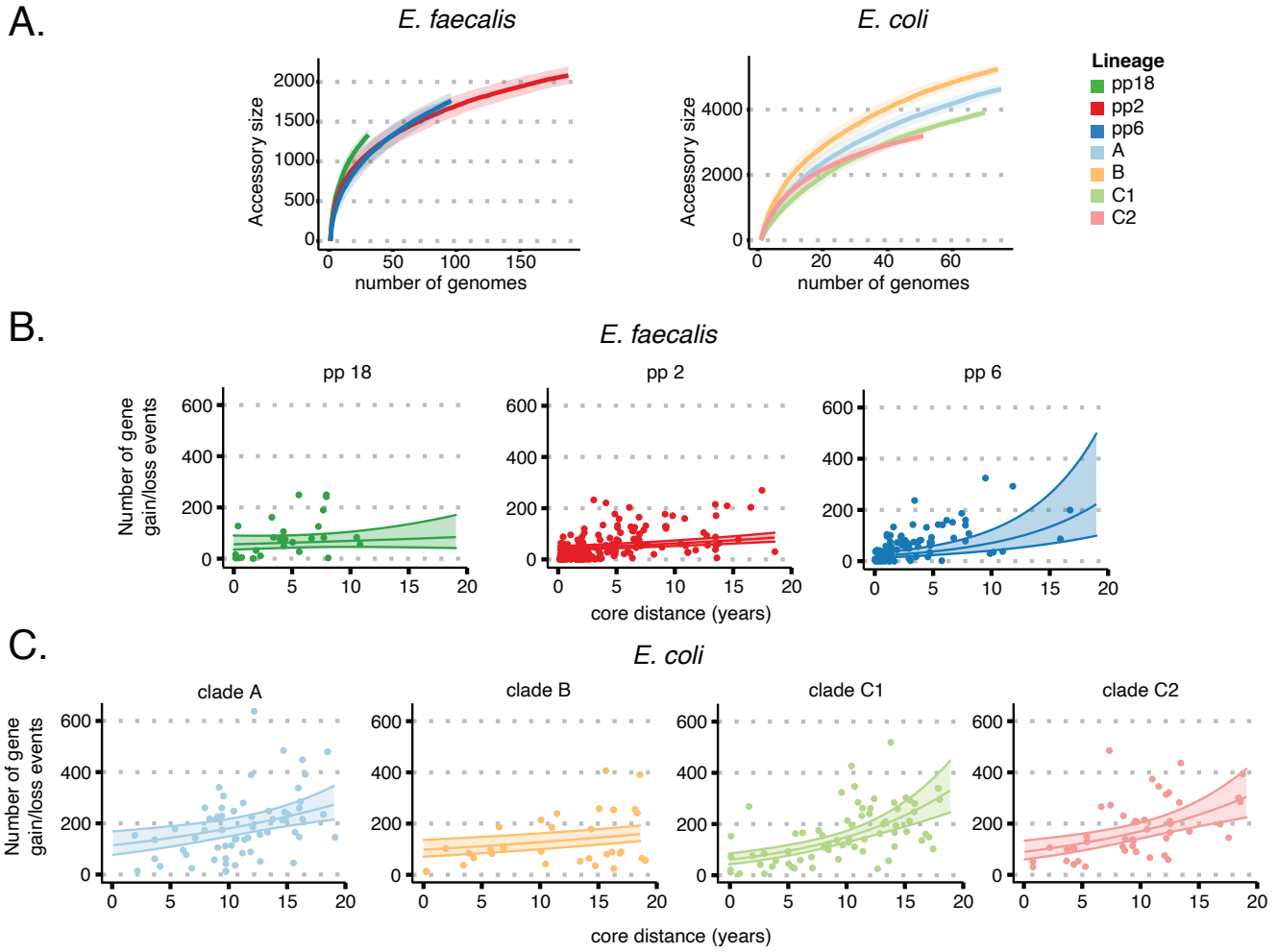

Fig. 4. A.) pangenome accumulation curves for the clades in the *E. faecalis* and *E. coli*-ST131 datasets. **B.)** *E. faecalis* Panstripe accessory versus core genome diversity plots with the underlying data indicated by points and the Panstripe model fit overlaid. **C.)** similar to B. but for the clades in the *E. coli* dataset.

faecalis. In contrast to the specialisation of each of the *E. coli* ST131 clades mediated by the gain and loss of specific MDR plasmids, there was not a significant difference between the *E. faecalis* clades and the *E. coli* clade B (40, 41). Our ability to detect smaller differences in the rates of gene exchange is limited by the large differences in the structure of the underlying phylogenies, which leads to increased uncertainty and a reduction in the statistical power to detect differences in these pairwise comparisons.

Interestingly, although we did not find a significant difference in the error rates between the *E. coli* and *E. faecalis* datasets, there was a significant difference in the dispersion parameters of the Panstripe GLM. This suggests that the number of genes involved in each gain and loss event differs significantly between the two species which is likely to be influenced by the different mechanisms driving HGT in the two species and the size of the MGEs (42). HGT in *E. coli*, is predominantly the result of phage interactions and the exchange of large F-plasmids (size on average > 100 kbp) while the total plasmid content of *E. faecalis* is generally lower (average $\sim$ 24 kbp) (35, 40). Therefore, *E. coli* has a substantial part of the accessory genome residing on large MGEs which have shaped the evolution of the subclades whereas the events of gain and loss for *E. faecalis* may involve smaller MGEs which allocate fewer genes.

Improved estimates of associations between phenotypes and pangenome evolutionary dynamics. The GLM framework used in Panstripe allows for other covariates and phenotypes of interest to be easily incorporated into the model and tested to identify significant associations with the rate of gene gain and loss. Common covariates of interest include whether lineages are associated with particular environments such as hospitals, drug resistance or invasive disease.

Previously, we have used the Finitely Many Genes model, as implemented in Panaroo, to estimate the rates of gene gain and loss in 51 major Global Pneumococcal Sequencing Clusters (GPCSs) (12, 23, 43, 44). The correlation between the estimated rates and the invasiveness of each lineage was estimated using Spearman's correlation coefficient. This approach identified an association between the rate of gene gain and loss and whether the lineage had a significant odds ratio of invasive disease (43). Although Panaroo significantly reduced the number of errors, this approach was still sensitive

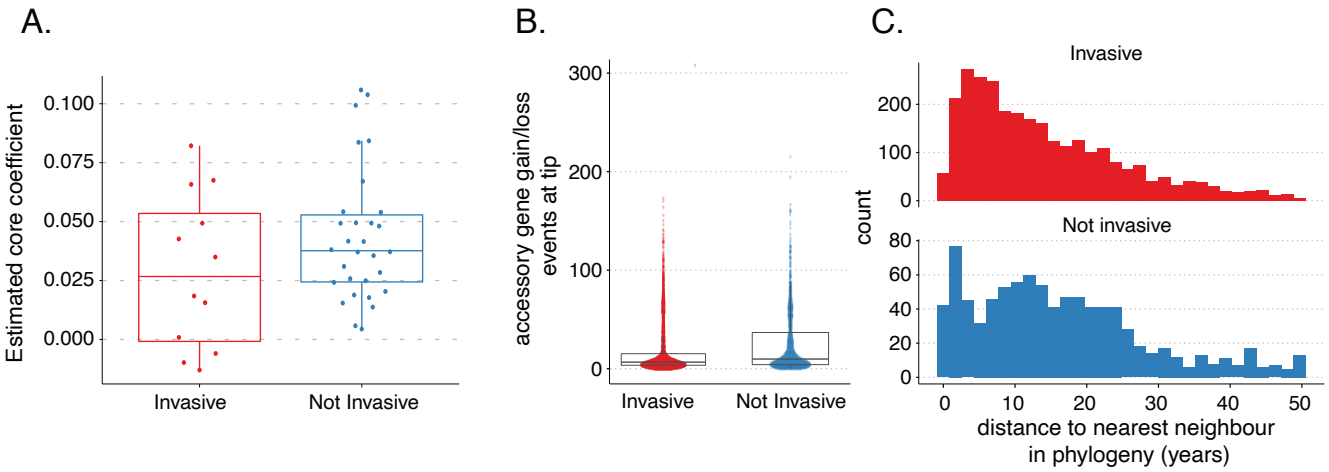

Fig. 5. A.) The estimated coefficient of the core genome parameter of the Panstripe GLM for each of the GPSCs. A higher value indicates an increased rate of gene gain and loss and invasive GPSCs were found to have a decreased rate of gene exchange. **B.)** The number of accessory genes found to have been either gained or lost at the tips of the core genome phylogenies in each GPSC. **C.)** Histograms of the pairwise patristic distance (in years) between isolates in invasive and non-invasive GPSCs.

to any remaining errors in the gene presence/absence matrix. To address this, we redid the analysis using the same gene presence/absence matrix inferred by Panaroo but with the Panstripe algorithm. Similar to our previous analysis, Panstripe identified a lower rate of gene gain and loss in GPSCs that had a significant odds ratio of severe disease ($p<1e-3$, Figure 5A) (12, 43, 44). This could be a signal of genome reduction which has been linked with pathogenicity across multiple divergence scales (45).

Unlike our previous analysis, Panstripe identified a significant association between whether a lineage was invasive and whether a branch was located at the tip of a phylogeny (Figure 5B). A potential explanation is that the annotation error rates differed between severe and nonsevere lineages. However, given that similar sequencing and annotation procedures were used for all genomes in the GPS project this is unlikely. Another explanation is that sampling bias or differences in the underlying population size of the lineages is driving the signal. Most invasive isolates, such as those expressing capsule like serotype 1, are known to have outbreak epidemiology (43). This could lead to a greater sampling of highly related pneumococcal strains from invasive lineages. The greater evolutionary time separating non-invasive lineages would thus allow for a higher number of unique gene exchange events to occur at the tips of the phylogenies, which are more heavily influenced by faster moving MGEs. This hypothesis is supported by looking at the pairwise patristic distance separating invasive and non-invasive isolates (Figure 5C). Here, invasive isolates are more closely related leading to a smaller number of rare unique genes being identified at the tips of the phylogeny. This highlights the utility of the Panstripe algorithm and helps to demonstrate its ability to account for both annotation errors, population structure and sampling biases.

Discussion

Determining the presence/absence of genes in prokaryotic genomes is a complex and error prone process. Annotation and clustering errors as well as sampling bias and differences in the underlying population structure of strains and species can all complicate the analysis of pangenome dynamics. To address these problems we developed Panstripe, an algorithm which is robust to both errors in the gene presence/absence matrix and the diversity of the underlying genomes. Panstripe is able to compare the gene exchange rates between pangenomes and determine if these differences are associated with the underlying core genome diversity, rare and erroneous genes occurring at the tips of a phylogeny or due to the average size of gene gain and loss events. The use of a GLM framework also allows for associations between gene exchange and covariates of interest to be investigated.

We found that methods that do not account for the diversity of the underlying genomes perform particularly badly including the commonly used pangenome accumulation curve and Heaps power law. This was most evident in the analysis of two gene presence/absence matrices obtained after running the Panaroo and Roary pipelines on the same dataset. The pangenome accumulation curve incorrectly indicated a diverse pangenome whilst Panstripe was able to use the additional core genome phylogenetic information to correct for the large difference in error rates of the two pangenome clustering tools.

Given a lack of an association between the core genome branch length and the accessory genome it is tempting to say that such a pangenome is ‘closed’. However, the binary classification of pangenome into ‘open’ and ‘closed’ can be problematic as it does not incorporate the evolutionary timescale being considered. For example, while there is unlikely to be any gene exchange in an outbreak of *M. tuberculosis*, there is evidence of pangenome variation across the Mycobacterium genus (46–48). Instead, we suggest that it is better to report whether the core and accessory genome variation is ‘coupled’ in that there is a significant association between the core and accessory genome diversity in a particular dataset.

While we have shown that Panstripe provides considerable improvements to the analysis of pangenome dynamics, it

does not implement a formal evolutionary model. Instead, we hope it provides a substantial improvement over the use of pangenome accumulation curves and allows for simple hypotheses to be tested. As more complex evolutionary models are proposed we expect that Panstripe will be used in a similar manner to TempEst — as a first check for signal prior to running more computationally intensive algorithms.

Panstripe is written in R and is available under the open source MIT licence from https://github.com/gtonkinhill/panstripe. The code used to generate the simulations and produce the analyses along with the gene presence/absence matrices and phylogenies is available from https://github.com/gtonkinhill/panstripe-manuscript.

Methods

Overview. The Panstripe algorithm takes a binary gene presence/absence matrix and an accompanying core genome phylogeny as input for each pangenome dataset being considered. Initially, the ancestral state of the presence of each gene at each node in the core genome phylogeny is inferred using either maximum parsimony or maximum likelihood methods. Using these estimates, the total number of gene gain and loss events on each branch of the phylogeny is calculated. A GLM framework is then used to compare the number of gene gain and loss events with the branch length of the core genome phylogeny. Terms are also included in the GLM to indicate the depth of the branch within the phylogeny and whether or not the branch occurs at the tips of the tree. These help to control for the reduced ability to observe gene gain and loss events at higher branches of the tree as well as errors in the gene presence/absence matrix respectively. Comparisons between pangenomes are made by including an additional categorical covariate in the GLM for the pangenome and investigating the interaction terms between this variable and the core, tip and depth terms.

Ancestral state reconstruction. Panstripe includes two methods for inferring whether a gene was present or not at each ancestral node of the core genome phylogeny. The default maximum parsimony approach, uses a version of Sankoff's dynamic programming algorithm adapted from the Castor R package to determine the ancestral states that correspond with the smallest number of state changes along the phylogeny (27, 28). Alternatively, a maximum likelihood based method assuming a fixed-rates continuous-time Markov model (Mk model) can be used (29). After reconstructing the ancestral state for each gene independently the total number of gene gain and loss events for each branch is found by taking the sum of events for each gene assuming that at most one change occurs between consecutive nodes.

While maximum likelihood based methods can provide improved estimates of the ancestral states in some instances (49), using an maximum-likelihood method effectively uses the branch lengths of the core genome phylogeny twice. Once in the ancestral state reconstruction and once in the regression. This can artificially inflate the correlation between the branch lengths and the number of gene gain and loss events. Consequently, we prefer to use maximum-parsimony. In practice, we have found that using either approach gives very similar results in most instances.

Tweedie generalised linear regression model. After inferring the number of gene gain and loss events, each branch of the phylogeny is subsequently treated as an independent data point. For each branch i, let g_i be the number of gene gain and loss events, l_i the core branch length, d_i the core genetic distance from the root of the phylogeny to the node at the start of the branch, and k_i be a binary variable indicating whether or not the branch occurred at the tip of the phylogeny.

We assume that the number of gene gain and loss events g_i follows a compound Poisson distribution. That is, the number of HGT events follows a Poisson distribution but the size of each event follows a Gamma distribution. This is important as HGT events frequently involve the acquisition or loss of multiple genes. To estimate the association between the number of genes gained and lost and the other covariates we make use of a Tweedie Generalised Linear Model implemented in the statmod R package (50). The Tweedie distribution generalises many distributions commonly used in GLMs including the Normal, Poisson and Gamma distributions depending upon the index power parameter p. For $1 < p < 2$, the Tweedie distribution is equivalent to a compound Poisson distribution (51).

A Tweedie GLM with a log link function assumes that the expectation of the ith response $\mu_i := E(g_i)$ is related to a vector x_i of covariates with corresponding coefficients b by

$$\begin{aligned} \log(\mu_i) &= x_i^T \beta \\ &= (1 + l_i + k_i + d_i + l_i k_i)^T \beta \end{aligned} \tag{1}$$

We include an interaction term between the core genome branch length and whether the branch is at the tip of the phylogeny to account for the fact that a gene can only be erroneously omitted if there is sufficient time for a gain event to have occurred.

The corresponding variance of g_i is given by

$$\mathrm{var}(g_i) = \mu_i^p \tag{2}$$

Using this framework, it is possible to perform standard hypothesis tests based on a Student t-distribution to determine whether each of the parameters is significantly associated with gene gain and loss. A significant association with the core branch length indicates that there is evidence of HGT at the upper branches of the phylogeny. The association with the 'tip' covariate can be driven by a combination of both the error rate in the inference of the gene presence/absence matrix and the acquisition and loss of rare genes that are seen only once. The depth parameter accounts for the potential loss in power to identify HGT at higher branches of the phylogeny.

Comparing pangenomes. Panstripe uses interaction terms to compare the relationship of the covariates with gene gain and loss between two pangenome datasets. While the use of a standard GLM framework would allow for the comparison of the inferred slope or β parameters between datasets, it assumes a single dispersion parameter. This effectively fixes the relationship between the rate of gene exchange and the size of each HGT event. This can be seen in the relationship between the Tweedie distribution parameters and the corresponding parameters of the Poisson and Gamma components of the Compound Poisson distribution

$$\lambda = \frac{\mu^{2-p}}{\phi(2-p)}$$
$$\alpha = \frac{2-p}{p-1}$$
$$\theta = \phi(p-1)\mu^{p-1}$$

Here, λ is the mean of the Poisson distribution and α and θ are the shape and scale parameters of the Gamma distribution respectively.

To relax this assumption, Panstripe optionally allows for the dispersion parameter to vary between pangenomes using the Double Generalised Linear Model (DGLM) framework described in Smyth, (32). The DGLM framework models the mean and dispersion using two separate GLMs with both being a function of the covariates. A maximum likelihood estimate of the parameters is then found by alternating between the two sub-models. Setting the pangenome being considered for branch i as a binary covariate n_i the DGLM used to compare pangenomes can be formulated as

$$\begin{aligned}\log(\mu_i) =& x_i^T\beta_1 \\ =&(1+l_i+k_i+d_i+l_ik_i+ \\ &d_in_i+k_in_i+l_in_i+l_ik_in_i)^T\beta_1\end{aligned} \tag{3}$$

$$\begin{aligned}\log(\phi) &= z_i^T\beta_2 \\ &= (1+n_i)^T\beta_2\end{aligned} \tag{4}$$

The significance of the inferred coefficients of the interaction terms can be used to determine whether the association between HGT and the depth, core and tip covariates differs significantly between pangenomes. When investigating the dispersion parameter, Panstripe uses the Likelihood Ratio Test to determine whether there is a significant benefit to accounting for a variable dispersion between two pangenome datasets. In some cases, such as when comparing lineages from the same species, it may be safe to assume that the relationship between the mean and dispersion is similar in both pangenomes. In this case, it can be preferable to fix the dispersion parameter to improve the statistical power of an analysis.

Identifying associations with additional covariates. The GLM framework used by Panstripe allows for other covariates that may influence pangenome dynamics to be considered. This could include whether or not a lineage is highly drug resistant, hospital associated or frequently linked to invasive disease. In general, this is most useful when many separate lineages from a large genome collection of a single species are available as is becoming increasingly common with the advent of large sequencing studies and the introduction of WGS as a routine service for public health surveillance (43, 44, 52). To investigate such associations, Panstripe replaces the binary pangenome covariate in equation 3 with the covariate(s) of interest. These can be logical, continuous or categorical.

Bootstrap confidence intervals. Panstripe estimates confidence intervals for each coefficient using the Bootstrap, by resampling each branch with replacement (53). While it is possible to use the GLM model to obtain confidence interval estimates, this makes a number of assumptions about the distribution of the coefficients and is more susceptible to outliers. Panstripe uses the boot R package to calculate these intervals which implements a number of commonly used methods for calculating the interval from the resampled set of coefficients (53, 54). In general, we have found the Bootstrap confidence intervals to provide similar estimates to that of the GLM model. However, in some cases differences have indicated a high sensitivity of the result to particular branches. Optionally, Panstripe also includes the possibility of estimating Bootstrap p-values using the confidence interval inversion method (55).

Simulations. To compare the performance of Panstripe we simulated core genome phylogenies using the ape package (56). The number of gene gain and loss events on each branch was then simulated using the simSeq function in the phangorn package (57). We make the simplifying assumption that each gain or loss event will involve the same set of genes and simulate the size of the set using a Poisson distribution as implemented in R. Errors in the pangenome matrix were simulated by randomly adding or removing single entries in the final gene presence/absence matrix. The number of errors for each genome was simulated using a Poisson distribution. All code to reproduce the analyses is available in the accompanying GitHub repository.

Data and materials availability

Panstripe is available under an MIT open source licence on GitHub https://github.com/gtonkinhill/panstripe (archived at 10.5281/zenodo.6404363). The code, phylogenies and gene presence/absence matrices used in our analyses are available at https://github.com/gtonkinhill/panstripe-manuscript (archived at 10.5281/zenodo.6404403).

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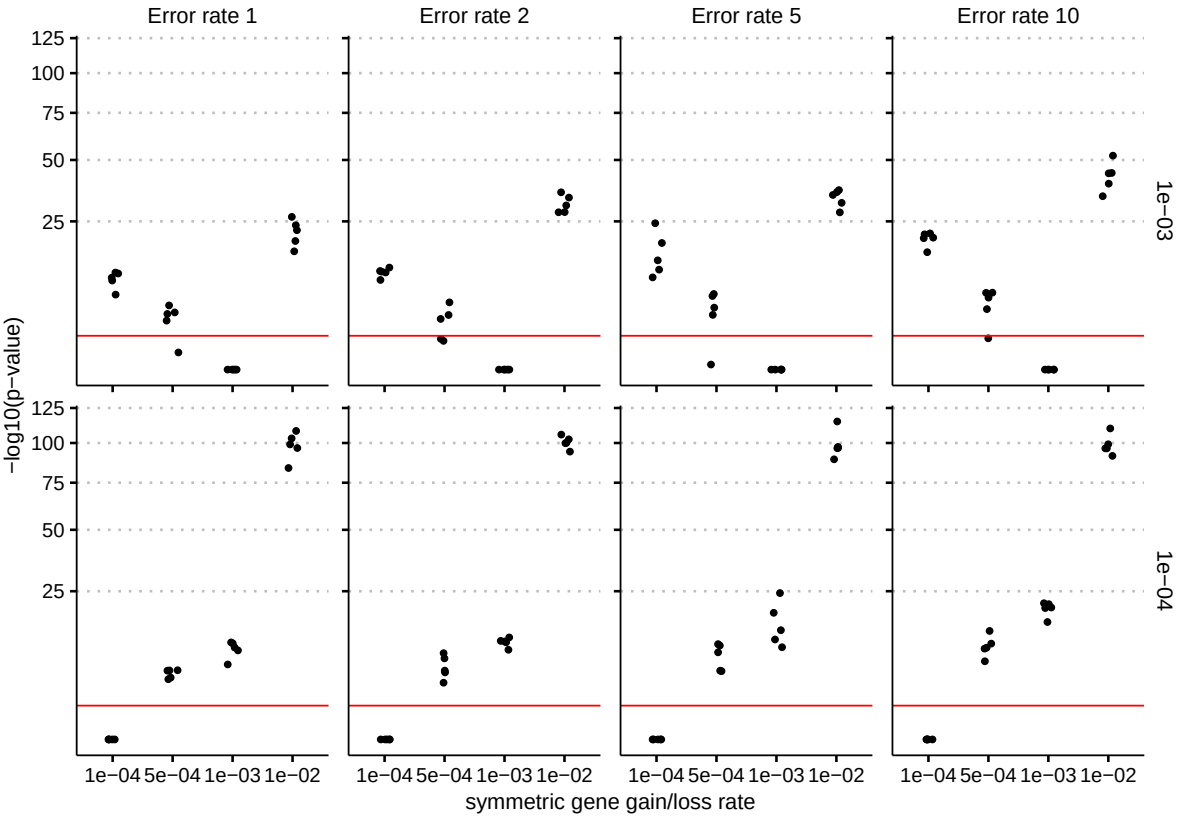
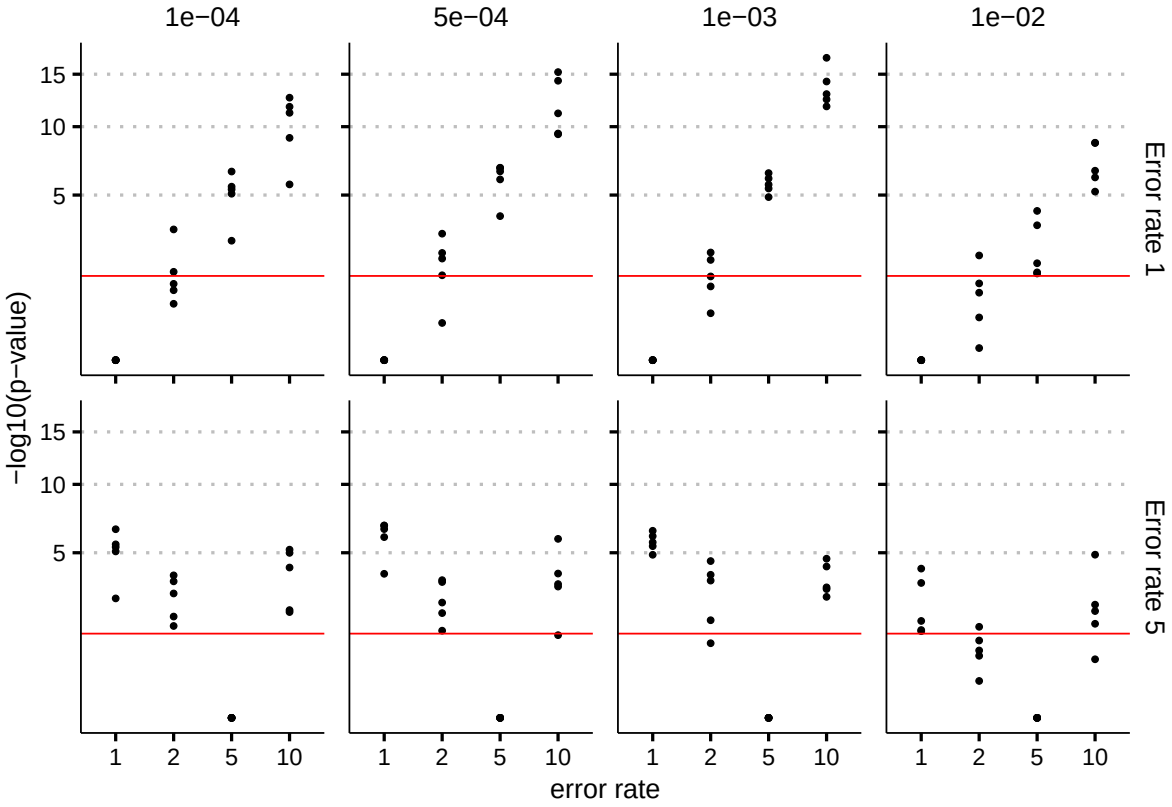
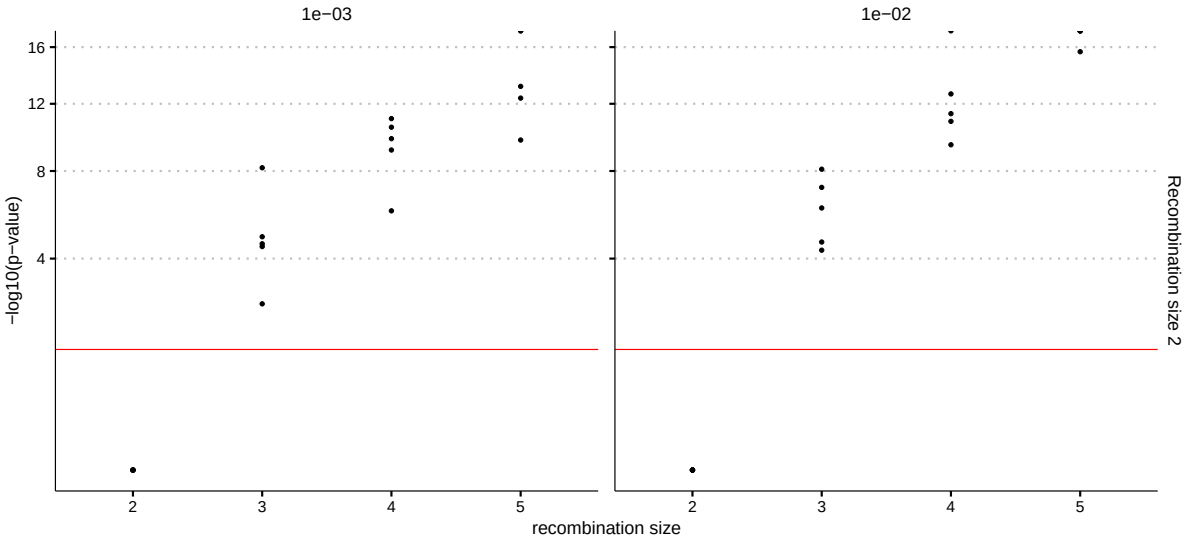
Supplementary Figures



Supplementary Figure 1. P-values for the core genome covariate obtained after running Panstripe on simulated data comprising four different symmetric gene gain/loss rates and four error rates. Five replicates were simulated for each parameter set. The top row of plots indicates the resulting p-values when comparing each dataset with the simulated gain/loss rate of 1e-3 whilst the lower row compares each simulated pangenome with those having a gain/loss rate of 1e-4. The horizontal red line indicates a p-value of 0.05. Points above this line are considered significantly different.



Supplementary Figure 2. P-values for the covariate indicating whether or not a branch occurred at the tip of a phylogeny. Five different error rates were simulated along with four symmetric gene gain/loss rates. Five replicates were simulated for each parameter set. The top row of plots indicates the resulting p-values when comparing each dataset with a dataset with a mean error rate of 1 while the bottom row shows comparisons with an error rate of 5. The horizontal red line indicates a p-value of 0.05 and points above this line are considered significantly different. Panstripe was less sensitive to differences in error rates when the gene gain and loss rate was very high ($\geq 1e-2$) which is rarely seen in practice.



Supplementary Figure 3. P-values obtained after performing a Likelihood Ratio Test to compare a model which allowed the dispersion parameter to vary between pangenomes with a model with a fixed dispersion parameter. The mean number of genes involved in a recombination event was simulated for five different parameter values and each compared with a mean size of 2. The horizontal red line indicates a p-value of 0.05 and points above this line are considered significantly different.

Publication	Method	Parameter
	Panstripe	core genome coefficient in GLM
Baumdicker et al. 2012	Panicmage (Infinitely Many Genes)	per generation rate of gene gain
Collins et al. 2012	Infinitely Many Genes (2 gene classes)	θ_1
Zamani-Dahaj et al. 2016	Finitely Many Genes	v
Tettelin et al. 2008	Heaps power law	α
Tettelin et al. 2005	accumulation curve	median number of genes found in 20 genomes

Supplementary Table 1. The parameters used to compare the results of each method on the simulated pangenome datasets.