Large scale automated phylogenomical analysis of bacterial whole-genome isolates and the Evergreen platform

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Abstract

Public health authorities whole-genome sequence thousands of pathogenic isolates each month for microbial diagnostics and surveillance of pathogenic bacteria. The computational methods have not kept up with the deluge of data and need for real-time results.

We have therefore created a bioinformatics pipeline for rapid subtyping and continuous phylogenomic analysis of bacterial samples, suited for large-scale surveillance. To decrease the computational burden, a two level clustering strategy is employed. The data is first divided into sets by matching each isolate to a closely related reference genome. The reads then are aligned to the reference to gain a consensus sequence and SNP based genetic distance is calculated between the sequences in each set. Isolates are clustered together with a threshold of 10 SNPs. Finally, phylogenetic trees are inferred from the non-redundant sequences and the clustered isolates are placed on a clade with the cluster representative sequence. The method was benchmarked and found to be accurate in grouping outbreak strains together, while discriminating from non-outbreak strains.

The pipeline was applied in Evergreen Online, which processes publicly available sequencing data from foodborne bacterial pathogens on a daily basis, updating the phylogenetic trees as needed. It has so far placed more than 100,000 isolates into phylogenies, and has been able to keep up with the daily release of data. The trees are continuously published on https://cge.cbs.dtu.dk/services/Evergreen

Keywords

Phylogenomics, WGS, subtyping, SNP, automation, epidemiology, outbreak investigation

Main

Epidemiological typing of bacteria is used by hospitals and public health authorities, as well as animal health authorities, to detect outbreaks of infectious diseases and determine trends over time. Traditionally, that includes culturing and isolating the pathogen, followed by species identification and subtyping using various conventional microbiological and molecular methodologies.

For outbreak investigation, it is necessary to place the infectious agent into a more discriminatory category than species, to establish links between cases and sources. Multi-locus sequence typing (MLST) has been a frequently used molecular subtyping method, where sequence types are assigned to the isolates based on the combinations of alleles for 6-10 housekeeping genes[1].

Whole-genome sequencing (WGS) has opened a new chapter in microbial diagnostics and epidemiological typing. WGS data can be used to determine both MLST types and serotype of several bacterial species[2,3]. Several studies for multiple bacterial species have shown the value of WGS for elucidating the bacterial evolution and phylogeny, and identifying outbreaks[4–6].

The use of WGS has enabled the unbiased comparison of samples processed in different laboratories, boosting surveillance and outbreak detection, but the methods for sharing and comparing a large number of samples have not been established yet[7,8]. Therefore, a number of national, regional and international initiatives have been launched with the aim of facilitating the sharing, analyses and comparison of WGS data[9–11].

Since 2012, the US Food and Drug Administration (FDA) has lead a network of public health and university laboratories, called GenomeTrackr. These laboratories sequence bacterial isolates from clinical and environmental samples and upload the data to the National Center for Biotechnology Information (NCBI). GenomeTrackr is restricted to foodborne pathogens and currently includes data from only seven such bacterial species. All raw WGS data are publicly shared, facilitating the collaboration between laboratories.[12] Furthermore, the NCBI Pathogen Detection pipeline[13] assembles the samples into draft genomes to predict the nearest neighbors and construct phylogenetic trees using an exact maximum compatibility algorithm[14]. This approach requires access to all of the raw data and very extensive computational power. In addition, no sub-species taxonomical classification has been implemented at this time.

Focusing on the same bacterial species as GenomeTrackr, PulseNet USA also has established procedures for use of WGS data for outbreak detection. In their vision, an extension of the highly successful MLST approach into a core-genome (cgMLST) or whole-genome (wgMLST) scheme, with genes in the order of thousand, would allow for sharing information under a common nomenclature. Meanwhile, all of the raw data could be kept locally. Only data from individual strains would have to be shared when further confirmation of an outbreak is required.[11] Consequently, a number of, at times conflicting, cg- and wgMLST schemes have been proposed for a limited number of bacterial species[15–22]. Moreover, few of the proposed schemes provide a definitive nomenclature of sequence types to go with the allele profiles. The existing schemes do not cover all of the potential allelic variation: a recent study showed, that for *Campylobacter jejuni*, that has maintained MLST schemes, only approximately 53% of the strains of animal origin could be assigned to an existing unique allelic profile[23]. Continuous curation of the hundreds of relevant bacterial species, that are known human, animal and plant pathogens, would require great effort. A centralized database for the distribution of the allele profiles and sequences would be also necessary. Furthermore, for comparable results, the same analysis pipeline or software should be used for the prediction of the allelic profiles.

The approaches mentioned above yield preliminary results and, in most cases, selected WGS data are further analyzed using single nucleotide profiling for outbreak detection. Here, genomic variants (single nucleotide polymorphisms (SNPs), insertions and deletions) are derived by aligning WGS reads to a reference genome. For

each bacterial species, custom single nucleotide profiling (SNP validation, cluster threshold determination, etc.) is necessary in order to achieve results that are biologically relevant and informative. The samples (of current interest and historical) included in the analysis and the reference genome are chosen on a case-by-case basis, usually based on subtyping results. Various SNP analysis pipelines are used by laboratories and research groups for inferring phylogenetic trees for isolates of interest[24–29]. For example, Public Health England developed and uses SnapperDB for outbreak detection without initial cluster analysis by cg- or wg-MLST. SnapperDB consists of tools to create a database of SNPs compared to a given reference sequence, and assign each isolate a SNP address based on single linkage clustering.[30]

We present here a whole-genome, single nucleotide-based method for subtyping and preliminary phylogenomic analysis, that circumvent the known limitations of current gene- and SNP-based approaches. PAPABAC carries out rapid and automated subtyping of bacterial whole-genome sequenced isolates and generates continuously updated phylogenetic trees based on nucleotide differences. We demonstrate two applications, a standalone version for local monitoring of bacterial isolates, and Evergreen Online, for global surveillance of foodborne bacterial pathogens. We also suggest a stable naming scheme for each isolate, making the results from the pipeline easier to communicate to others. To the best of our knowledge, no such tool exists at the moment.

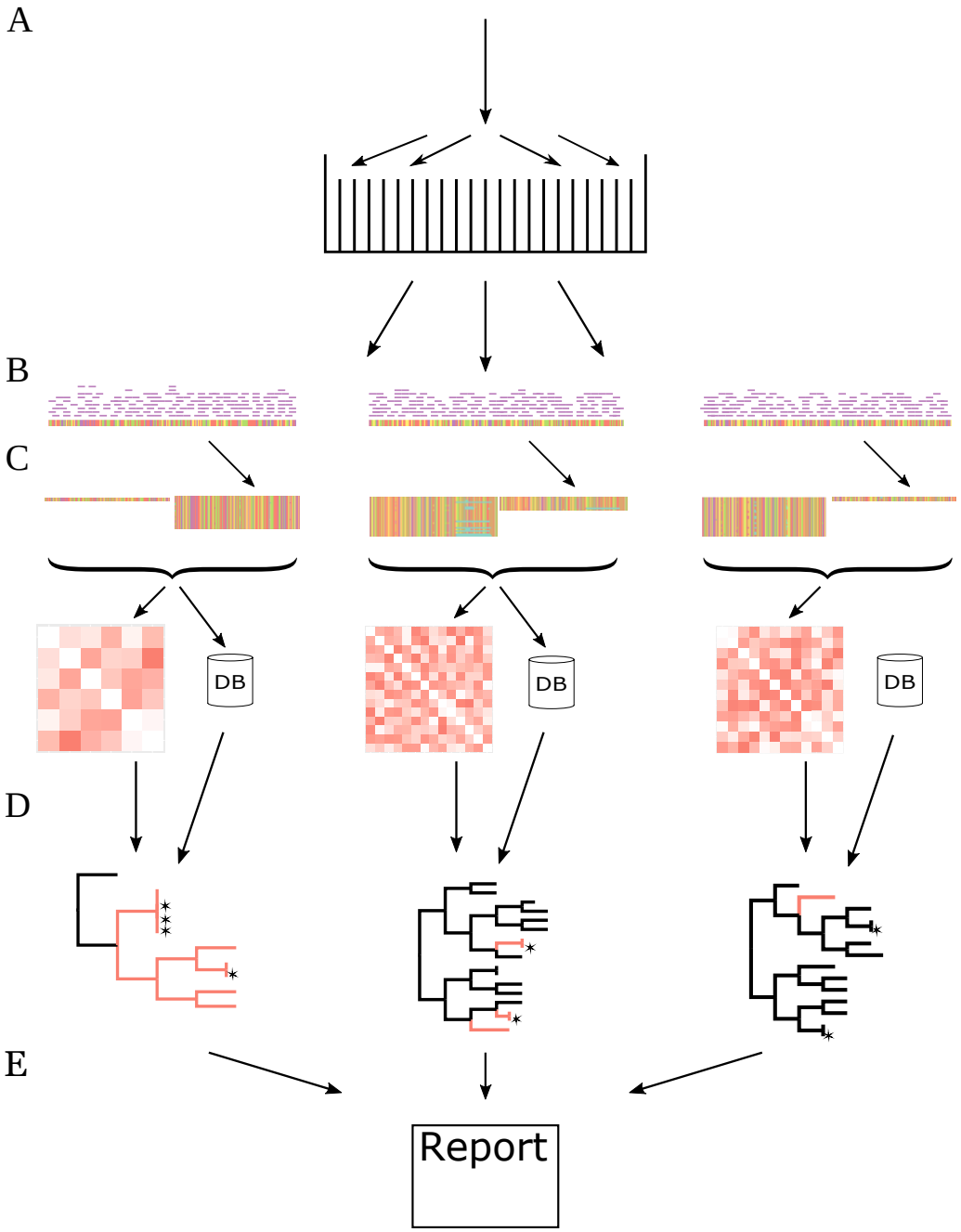

Figure 1 Overview of PAPABAC. (A) The input raw read files are classified into sets based on k-mer similarity to NCBI RefSeq complete prokaryotic chromosomal genomes. (B) The raw reads are mapped to the reference genome and a consensus sequence is generated via strict statistical evaluation ($p < 0.05$) of the mapped bases in each position. (C) The resulting consensus sequences are of equal length in each template set. The new isolates in each set are clustered to the non-redundant isolates already in the set if the pairwise nucleotide difference based genetic distance is less than 10. The remaining new isolates undergo the same clustering process. (D) Pairwise genetic distance between all non-redundant isolate in the set is used as input for neighbor-joining algorithm. If there are less than 600 non-redundant isolates in a set, an approximately maximum likelihood phylogenetic tree is also inferred based on the consensus sequences (red: new isolates). The clustered isolates are placed back onto the trees with 0 distance to the cluster representative (marked with an asterisk). (E) The information about the acquired isolates, the sets, the clusters, and the phylogenetic trees is stored in SQLite databases, which are queried once all sets with new isolates are processed to output the results to the users.

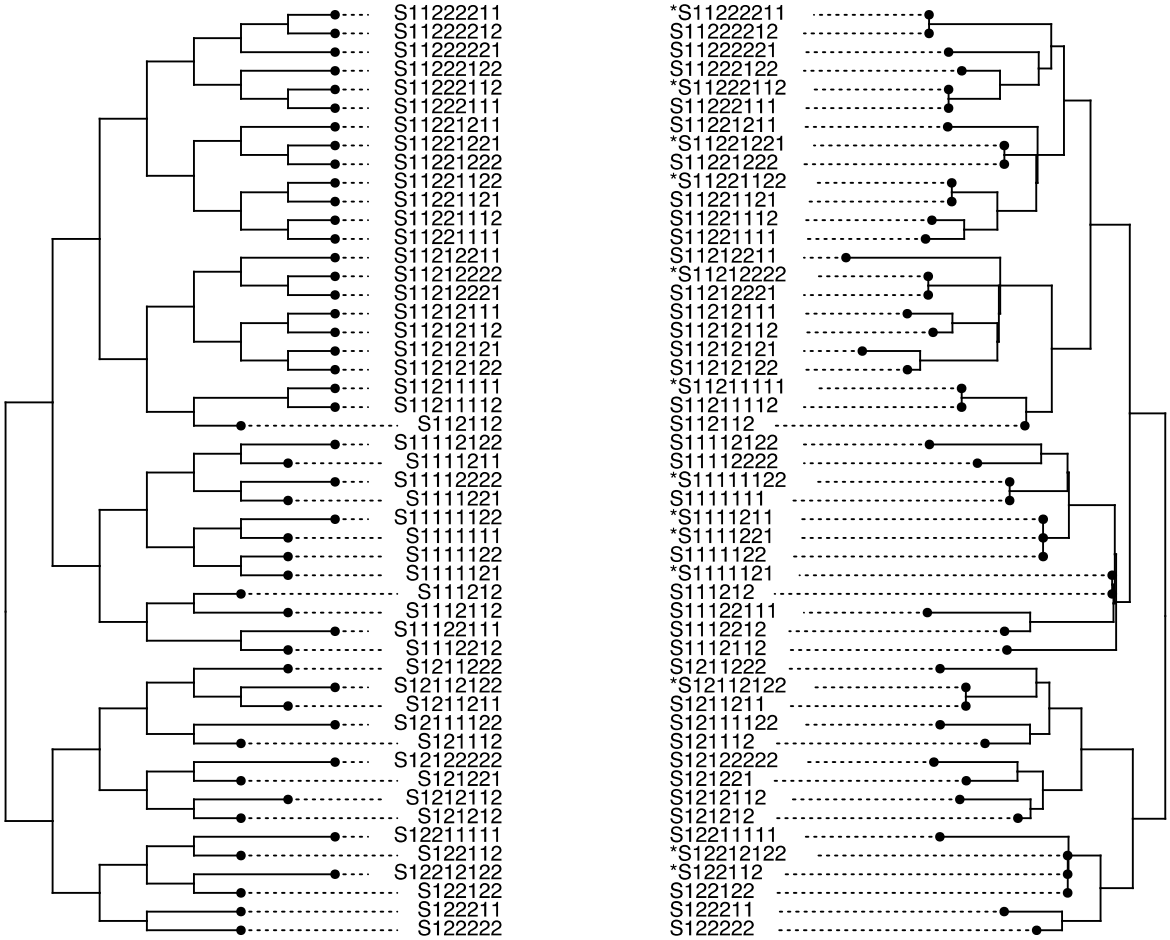

Figure 2 Comparison of the ideal tree (left) to the PAPABAC maximum likelihood tree made of the in vitro experiment dataset[31] Taxa with an asterisk were clustered together with the taxa in the same clade.

Results

Pipeline for automated phylogenomic analysis of bacterial whole-genome sequences (PAPABAC)

We developed PAPABAC (Figure 1), a phylogenomic pipeline for the automated analysis of bacterial isolates, that needs no additional input besides WGS data (fastq files) and generates clusters of closely related isolates. PAPABAC first matches the isolates to complete bacterial chromosomal genome reference sequences with greater than 99.0% sequence identity and a minimum average depth of 11. These reference sequences serve as templates for the alignment of the raw reads. The aligned bases at each position are statistically evaluated to determine the consensus sequence, as previously described for a nucleotide difference method[32]. Positions that do not fulfil the significance criteria remain ambiguous, get assigned "N", and disregarded during the pairwise genetic distance calculation. These steps ensure that there is high confidence in the consensus sequence that is the basis of the genetic distance estimation.

The pipeline retains analysis results in such a manner that input is added to the previously processed data. The phylogenomic analysis is carried out on the current input and the previously found non-redundant isolates (singletons and cluster representatives). The genetic distance is estimated in a pairwise manner, comparing the given two sequences for all non-ambiguous positions, i.e. positions where none of the two sequences have an "N" assigned. The distances between the previously processed runs are stored on disk, saving computational time, and only the distances to the new isolates are computed in a given run. A clustering step during the genetic distance calculation forms clusters of closely related isolates and reduces the number of similar sequences in each set, and thereby also reducing the computation time. After identifying a non-redundant isolate and a closely related isolate to it, the one previously deemed non-redundant will be the cluster representative and kept, while the clustered one will be omitted from the subsequent runs of the pipeline. However, the information about the clustering will be added to a database and the clustered isolate will be placed on the inferred phylogenetic tree. The cluster representatives remain constant through the subsequent runs of the pipeline, and the clusters only increase in size if new isolates are clustered with the representative. Therefore, each cluster is stable and can be reliably identified by the template name and the identifier of its cluster representative.

The pipeline can be run on on a computer with 8 Gb RAM and Unix system. The computational time is reduced compared to re-running the whole analysis each time new samples are added, even without parallelisation (Figure S1).

PAPABAC was benchmarked against three SNP pipeline benchmarking datasets. An *Escherichia coli in vitro* evolution experiment dataset[31] provided 50 closely related samples on a short temporal scale with less than 100 nucleotide differences across the dataset. The PAPABAC maximum likelihood (Figure 2) and neighbor-joining (Figure S2) trees were comparable to the ideal phylogeny of the *in vitro* experiment dataset. The algorithm clustered together 7 out of 10 samples with the same ancestor that were taken on the same day and presumably had less than 10 nucleotide differences between them.
Benchmarking against the *Campylobacter jejuni* (Figure S3A) and the *Listeria monocytogenes* (Figure S3B) datasets from Timme et al.[33], PAPABAC correctly clustered the related outbreak strains (colored) and the outgroups, where the genetic distance was below the clustering threshold. The topologies of the maximum likelihood phylogenetic trees closely resembled the tree topologies given.

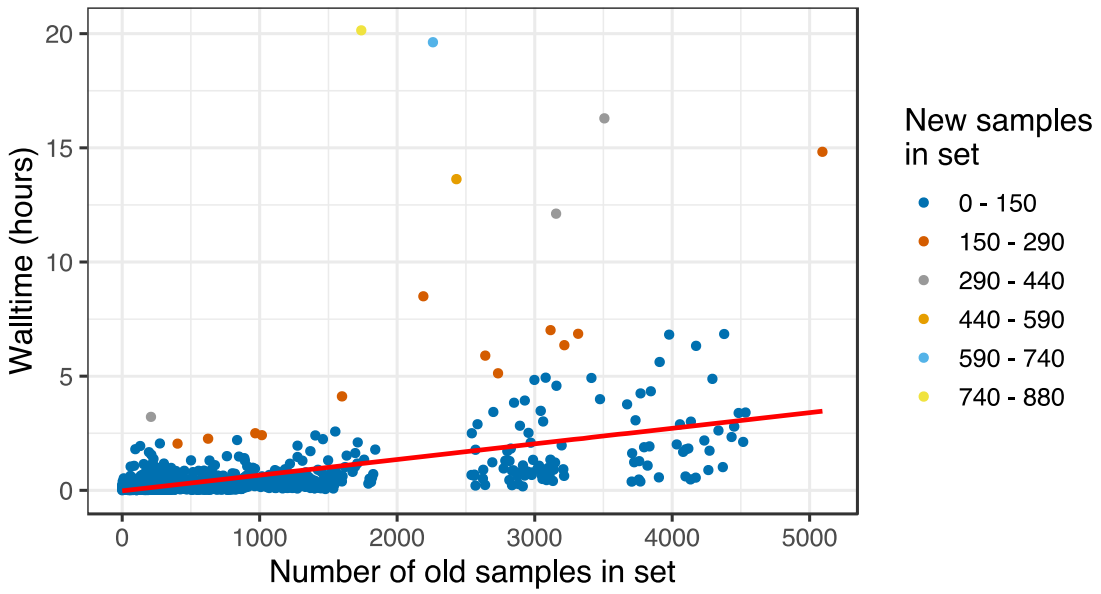

Figure 3 Time requirement of the phylogenomic analysis for given number of non-redundant and new strains, on 20 CPUs.

Evergreen Online for surveillance of foodborne bacterial pathogens

Evergreen Online was built on PAPABAC. Raw WGS data files of five major foodborne pathogens (*C. jejuni*, *E. coli*, *L. monocytogenes*, *Salmonella enterica*, and *Shigella* spp.) are downloaded daily from public repositories with the aim of global surveillance of potential outbreaks worldwide. The inferred phylogenetic trees and information about all of the isolates in the system are available and searchable on the website (http://cge.cbs.dtu.dk/services/Evergreen).
The platform has been available since October 1st 2017, with logs reliably saved since October 28th 2017. The number of raw read files downloaded fluctuates with the work week of the public health laboratories. On busier days, more than 800 isolates are downloaded. The average number of isolates downloaded is 418. Downloading and mapping to the reference genomes take 130 minutes on average, with the majority of the time spent on downloading. Alignment of the raw reads and the generation of the consensus sequences takes on average 9 minutes per isolate. The computing time for the template sets is dependent on the number of non-redundant and new sequences in each set, but in most cases even the slowest is finalized within five hours (Figure 3).

As of June 26th 2018, the pipeline downloaded 82,043 isolates. Out of these, 63,276 isolates have been mapped to references with at least 99.0% identity and average depth of 11 (Figure S4A). The majority of the isolates were typed as *Salmonella enterica* (59.1%), followed by *Escherichia coli* (19.4%) (Figure S4B). The two largest template sets are *S.* Dublin and *S.* Typhimurium serovars, with both close to 9,500 isolates in total. After the homology reduction there were 3,216 and 5,093 non-redundant sequences in these sets, respectively. On average, 67% of the sequences are non-redundant in the template sets, while the E. coli template sets are the most diverse and the *Listeria monocytogenes* ones are the least diverse (Figure S4C). There were 122 isolates predicted to have a type not specified by the query (Table S1). Of these, 14 isolates were mixed samples, composed of both the queried and the non-queried organisms.

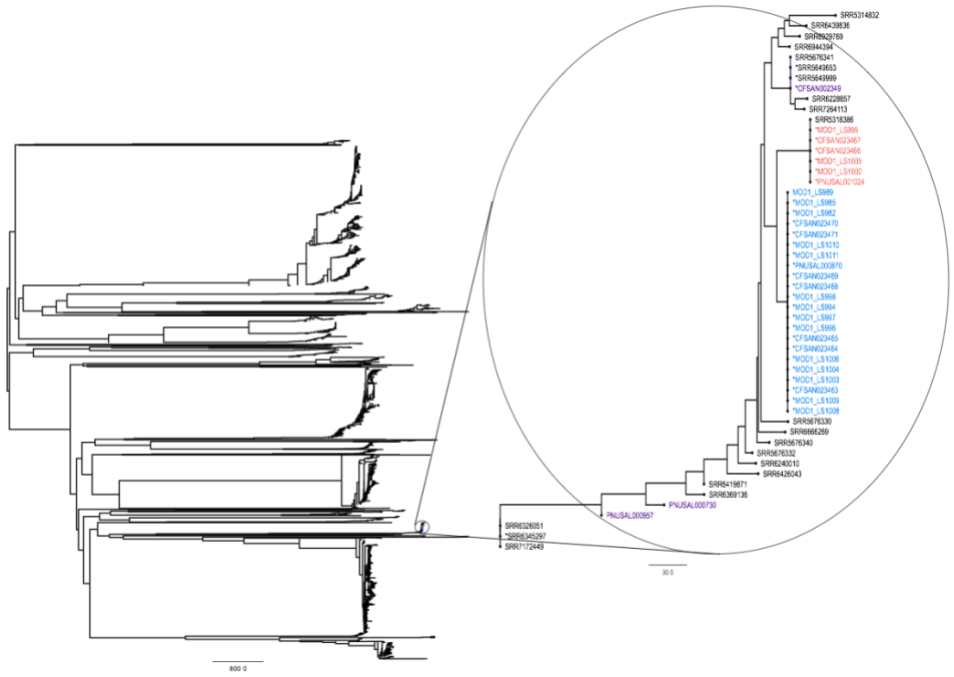

Figure 4 Neighbor-joining tree for the Listeria_monocytogenes_07PF0776_NC_017728_1 set after the samples of the L. monocytogenes dataset were added. Isolates colored in concordance with Figure S3B

The *L. monocytogenes* SNP pipeline benchmarking dataset[33] was added to the template set (Listeria_monocytogenes_07PF0776_NC_017728_1) of the corresponding reference genome in Evergreen Online, to test the sensitivity and accuracy of the clustering in large datasets. This template set at that moment contained more than 2400 isolates, of which 1398 were non-redundant. The isolates were placed onto a clade of a clonal lineage. The outbreak and outgroup isolates were separated in concordance with the ideal phylogeny (Figure 4). The smaller clade of outbreak samples clustered to a sample (SRR538386) of an environmental swab in 2014, from California, USA.

Isolates that were presumed to be from an *E. coli* O157:H7 outbreak were selected for the comparison of Evergreen Online and the NCBI Pathogen Detection platform (NCBI-PD). They were located on the Escherichia_coli_O157_H7_str_Sakai_chromosome_NC_002695_1 neighbor-joining (NJ) tree from Evergreen Online and the PDS000000952.271 SNP cluster tree from NCBI-PD. The labelled isolates appeared in three clusters on the NJ tree. There were 19.9 nucleotide differences between the yellow and the red cluster representatives and 12.6 nucleotide differences between the yellow and the blue cluster representative. On the PD tree, the isolates marked with red circles were on the same clade, while the ones marked with blue and yellow were intermixing on clades that were, at most, 15 compatible characters apart (Figure 5).

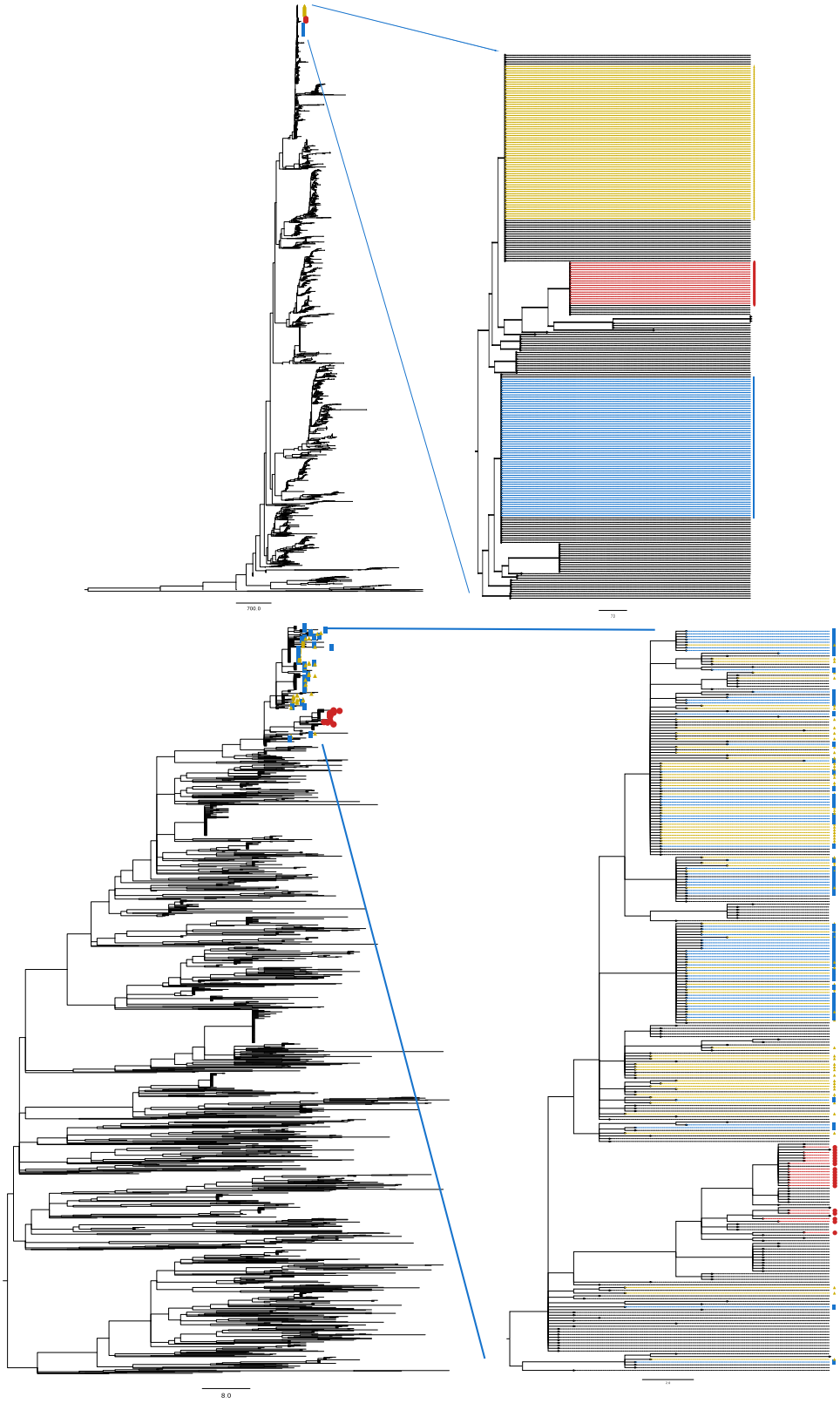

Figure 5 Selected isolates in the Escherichia_coli_O157_H7_str_Sakai_chromosome_NC_002695_1 NJ tree (top) and on the PDS000000952.271 SNP cluster maximum compatibility tree (bottom). The three largest clusters of the selected samples on the NJ tree are labelled with yellow, red and blue dots. These isolates were marked with the same labels on the NCBI-PD tree. The red labelled ones are on a single cladeon the PD tree, while the blue and yellow isolates are mixing on two other clades.

Discussion

Whole-genome sequencing, performed alongside the traditional methods in routine microbiology, yields hundreds to thousands of WGS isolates yearly in hospital, public health and food safety laboratories. This amount of data is overwhelming for many, and there is a lack of methods to generate a quick overview and help prioritize resources. The timely analysis of the sequencing data would allow the detection of more bacterial outbreaks and aid the prevention of further spread. However, lack of human and computational resources for this demanding task often hampers the prompt procession of the data. Automating the initial subtyping phase would facilitate the start of an outbreak investigation. PAPABAC offers rapid subtyping for a wide range of prokaryotic organisms: the supplied database covers all bacterial subtypes with complete genomes present in NCBI RefSeq. Further reference genomes could be added to increase the covered sequence space, but the active curation of the reference database is not required for routine usage. The selection of the reference sequence for the phylogenomic analysis is fast and robust. It is independent of pre-assumptions about the isolates. Misclassification during previous analysis does not introduce errors into the downstream analysis. Contamination from another species is discarded during the consensus sequence generation. The subtyping step via k-mer based mapping to a close reference also serves as a sequencing quality control measure, because low-quality sequencing runs will typically result in isolates with low identity to any reference and/or low depth. These isolates do not progress further to the phylogenomic analysis, as they would not yield reliable results.

The phylogenomical analysis performed on the template sets has higher discriminatory power than cg- or wg-MLST. The underlying nucleotide difference method was validated in five different studies[6,31,32,34,35]. By using all positions in the consensus sequences for estimating the genetic distance, instead of considering only selected loci, we ensure a high level of sensitivity, as we also include mutations that occur between genes.

The clustering step during the genetic distance calculation was introduced in order to reduce the homology in the template sets and thus reduce the computational burden as the template sets increase in size. However, the clustering threshold of 10 nucleotide differences also constructs informative clusters of highly similar isolates. Benchmarking with the *E. coli in vitro* evolution experiment dataset (Figure 2) showed that the algorithm was capable of correctly clustering isolates that were derived from the same ancestor, while distinguishing them from other closely related strains. The same sensitivity was demonstrated on empirical outbreak datasets (Figure S3), where the pipeline clustered the outbreak-related strains and separated them from the outgroup strains. Both the maximum likelihood inferred and the neighbor-joining trees placed the outbreak strains correctly in the phylogeny. These results show, that PAPABAC provides quick and reliable information about the close relatives of an outbreak strain to provide candidates to perform a more thorough analysis on.

The design of PAPABAC means that once an isolate passed the homology reduction step, it will be present in the subsequent runs of the pipeline. When an incoming isolate is highly similar to a non-redundant one, the more recent will be the one that is clustered, added to the database and removed from further runs. Hence, the cluster representatives and clusters are robust to the addition of new data to the analysis. Therefore, PAPABAC yields a stable and communicable name for the clusters, comprised of the template name and the cluster representative.

Evergreen Online has been steadily processing WGS data of foodborne bacterial pathogen isolates collected worldwide in real time (Figure S4A). It has been able to keep pace with the flow of the generated data that mainly came from public health and food safety laboratories. Excluding the download time and the optional maximum likelihood based phylogenetic inference, the whole analysis is done in less than a day, even for

241 template sets with thousands of isolates (Figure 3). This turnover time facilitates quick response in a potential
242 outbreak scenario.

243 The isolates are not distributed equally across the templates in the system (Figure S4B). Out of the five queried
244 species, *S. enterica* isolates are disproportionally represented. Sequences in the *S.* Dublin and the *S.*
245 Typhimurium LT2 template sets comprise in total approximately half of the *S. enterica* isolates. The sequence
246 diversity in the template sets is varied, but the homology reduction on the template sets reduces the number
247 of sequences approximately by a third, significantly decreasing the computational time. The *L. monocytogenes*
248 template sets were the least diverse, which could be due to sampling bias: bacteria that are present in the
249 environment are routinely sampled from food production sites multiple times, producing highly similar
250 sequences, that are then removed from the ongoing analysis. We also tested how a large number of sequences
251 already present in a template set would affect the ability of the pipeline to discriminate between samples
252 (Figure 4). The template set that corresponded to the stone fruit *L. monocytogenes* outbreak dataset reference
253 had more than 1,000 non-redundant isolates, which was ideal for the test analysis. The isolates that were part
254 of the same outbreak clustered together and formed the two expected outbreak clusters, despite the
255 confounding presence of the sequences already in the template set. The smaller clade, however, had a
256 different cluster representative when using all data for the template set, compared with analysis of the
257 outbreak data alone: an environmental sample, that could be related to the outbreak, as it was sampled from
258 the same US state and year (California, 2014) as the samples in the outbreak dataset. These findings indicate
259 that the pipeline is capable of identifying closely related samples, however it is necessary to conduct
260 epidemiological analysis and apply other knowledge when interpreting the results.

261 Evergreen Online allows for automated selection of closely related isolates out of thousands, which is also the
262 objective of NCBI-PD. *E. coli* isolates, situated on three clusters in Evergreen Online and supposedly from an
263 outbreak, were located in NCBI-PD and their placement in the SNP cluster tree was compared to the Evergreen
264 Online tree (Figure 5). One cluster (red) was in agreement between the two platforms, and samples from the
265 other two (yellow and blue) clusters were intermixing on a clade on the NCBI-PD tree. The nucleotide
266 difference counts between these samples are low and the differences between the phylogenomic methods
267 could lead to differences in the finer details of the inferred phylogenies. The homology reducing clustering in
268 Evergreen Online means that any sample in the cluster is less than 10 nucleotide differences from the cluster
269 representative, however, the differences between the samples could amount to 18 nucleotides. The
270 compatible character distances on the NCBI-PD tree between the mixed samples are less than 18 characters.
271 Taking this into account, the observed distribution of the yellow and blue labeled samples is concordant with
272 our results.

273 *Table 1 Comparison of pipelines for large-scale surveillance for pathogenic bacteria*

	SnapperDB	NCBI-PD	PAPABAC
For a wide range of bacterial species	x	-	x
Requires only raw sequencing reads as input	-	x	x
Whole-genome based	x	x	x
Assembly-free	x	-	x
Quality control steps	x	x	x
Automated phylogenomical analysis	-	x	x
Stable clustering of samples across runs	-	-	x
Communicable nomenclature for subtype and cluster	X	-	x
Open source	x	-	x

In summary, we developed PAPABAC with the aim of rapid subtyping and continuous phylogenomical analysis on a growing number of bacterial samples. PAPABAC overcomes limitations of cg- and wg-MLST approaches by tolerating genomic variation during subtyping, but providing greater sensitivity during the phylogenomical analysis. It was benchmarked on datasets created for testing SNP-based pipelines, and was proved to be accurate in discriminating between outbreak related and non-related samples. The software is open source and fulfills expectations put to WGS-based surveillance pipelines (*Table 1*). Evergreen Online, an application made for the global surveillance of foodborne bacterial pathogens, demonstrates the accuracy, speed, stability and practicality of PAPABAC on thousands of samples via an on-going analysis, where the results are published online.

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Methods

Bioinformatics pipeline: PAPABAC

The pipeline takes raw whole-sequencing reads (fastq files) as input. Matching reference sequences (templates) in our reference database, that have greater than 99.0% identity, are identified for the isolates using 16-mers via KMA[36] in sparse mode. Multiple templates are accepted, if they meet the criteria, allowing for the procession of mixed samples. Information about the runs and their templates are inserted into the main SQLite database. The isolates are grouped into sets according to the matched templates. The next steps are performed in these sets in parallel. The isolate reads are mapped to the template using the mapping algorithm

392 of NDtree[32], yielding equal-length consensus sequences. The Z-score threshold for accepting a base is set to
393 1.96.

394 Genetic distance based on nucleotide difference is calculated pairwise between the previous, non-redundant
395 isolates and the new isolates. Positions with ambiguous bases are discarded. The new isolates are clustered to
396 the non-redundant ones with a threshold of 10, in order to reduce the homology in each set and form
397 informative clusters. In this step, the non-redundant isolate is prioritized over the new isolate and becomes the
398 cluster representative. After the clustering, the remaining new isolates are clustered together with the
399 Hobohm 1 algorithm[37]. In this case, the cluster representative is the one that has already passed the
400 redundancy threshold. The information about new or extended clusters is saved to the main SQLite database. A
401 distance matrix is constructed for all non-redundant isolates and saved to disk for use in the next run. A
402 distance-based phylogenetic tree is inferred by neighbor-joining[38,39]. If there are less than 600 non-redundant
403 isolates in the set, then a whole-genome based approximate maximum likelihood phylogenetic tree is also
404 inferred using IQ-tree[40], where the neighbor-joining tree is the starting tree and the GTR nucleotide
405 substitution model is used. The clustered isolates are placed back onto the clades with zero distances to the
406 cluster representative. Their tip labels start with an asterisk. The information about the trees is saved to the
407 main SQLite database.

408 When all the phylogenetic trees with new isolates have been inferred, then the main SQLite database is
409 queried for the list of all isolates, their templates, cluster representatives (if there is any) and the latest
410 phylogenetic tree they are on. This information is printed to a tab-separated file.

411 Scripts and installation instructions are available on bitbucket:
412 https://bitbucket.org/genomicepidemiology/evergreen

413 **Online Evergreen platform**

414 A query is made to the National Center for Biotechnology Information (NCBI) Sequencing Read Archive (SRA)
415 for the newly published Illumina paired-end sequenced isolates of *Campylobacter jejuni*, *Escherichia coli*,
416 *Listeria monocytogenes*, *Salmonella enterica*, and *Shigella spp*. on a daily basis. Fastq files of raw sequencing
417 reads and the corresponding metadata (collection date, location, institute, source, etc.) are acquired either
418 from SRA or from the European Nucleotide Archive (ENA). The downloaded isolates are the input to PAPABAC.
419 The metadata are saved in the main SQLite database, and added to the tip labels on the phylogenetic trees.

420 Once all instances of the second wrapper script have finished, then the SQLite databases are queried for the list
421 of available phylogenetic trees (the maximum likelihood trees preferred over neighbor-joining ones), changes
422 in the clusters and the list of all isolates in the system, which is then used to update the website.

423 **Architecture**

424 The pipeline is written in Python 2.7 and Bash in Unix environment. In addition to the standard Anaconda
425 Python 2.7 packages, it also requires ETE Toolkit v3.0[41] and Joblib v0.11 (https://pythonhosted.org/joblib)
426 packages to be installed. Neighbor program from the PHYLIP package v3.697
427 (http://evolution.genetics.washington.edu/phylip.html) and IQ-tree v6.0[40] are used for the phylogenetic tree
428 inference. The SQL database management is performed with SQLite v3.20.1 (https://www.sqlite.org).

429 The two main parts of the pipeline have their own wrapper scripts. PAPABAC can be run on a personal
430 computer with as few as four cores.
431 Evergreen Online is running on a high-performance computing cluster, utilizing the Torque (Adaptive
432 Computing Inc., USA) job scheduler. The first wrapper is run in one instance on 20 cores, meanwhile the second

433 wrapper is run once on 20 cores for each template that has at least one new run, in a parallel fashion. When all
434 of these instances are finished running, a Bash script is launched to collect the information from the SQL
435 database, the website is updated and the job for the next day is scheduled.

436 **Reference database**

437 The reference sequences are complete prokaryotic chromosomal genomes from the NCBI RefSeq database.
438 Homology reduction was performed at a 99.0% sequence identity threshold with the Hobohm 1 algorithm. The
439 curated NCBI prokaryotic reference genomes were given priority in the process. The reference sequences and
440 the classification database could be downloaded via ftp
441 (ftp://ftp.cbs.dtu.dk/public/CGE/databases/Evergreen/).

442 **Website**

443 The phylogenetic trees are interactively visualized on the website (https://cge.cbs.dtu.dk/services/Evergreen/)
444 using the Phylocanvas API (http://phylocanvas.org). The isolates and clusters can be searched by SRA run ID,
445 which allows the quick localization of the clusters that increased in size via their cluster representative.

446 **Computational time comparison of continued phylogenomic analysis**

447 101 samples from the Escherichia coli in vitro evolution experiment dataset by Ahrenfeldt et al. were batched
448 according to their sampling time. The parallelization in PAPABAC was disabled. The traditional method meant
449 that the analysis was carried out on all the samples up to the given batch, starting anew each time, but using
450 the same scripts as PAPABAC.

451 **Benchmarking of PAPABAC with the Escherichia coli in vitro evolution experiment dataset by Ahrenfeldt et**
452 **al.**

453 The last samples in each lineage were selected for the benchmarking. Therefore, the benchmarking dataset
454 constituted 50 tips on the ideal phylogeny. These samples were batched according to their sampling time (6th,
455 7th and 8th day). The batches were processed by PAPABAC chronologically. The pipeline was run with the
456 default parameters. Both maximum likelihood and neighbor-joining trees were inferred.

457 The phylogenetic trees inferred on all 50 isolates were trimmed for the reference sequence and compared with
458 the ideal phylogeny using the phytools R package[42].

459 **Benchmarking of PAPABAC with datasets from Timme et al.**

460 Each dataset was downloaded with the provided script into a distinct directory. The pipeline was run
461 individually on the datasets with default parameters. If the isolates were mapped to more than one template,
462 the phylogenetic trees of the template set with the highest number of isolates were evaluated. The maximum
463 likelihood trees were visually compared to the ideal phylogenies and checked for the distribution of the isolates
464 amongst the clades.

465 **Comparison with the NCBI Pathogen Detection platform**

466 *Escherichia coli* isolates were queried from the SQL database of Evergreen Online (EO) for the period of 2018-
467 03-15 and 2018-06-01, corresponding to a multistate outbreak of *E.coli* O157:H7 in the USA[43]. These samples
468 were subtyped using traditional MLST[2], as it was assumed, that the sequence type with the most isolates would
469 also include the outbreak samples. Sequence type 11, which is commonly corresponds to the O157:H7
470 serotype, was selected for further analysis. The corresponding samples and their SNP clusters were found in
471 the NCBI-PD platform. The phylogenetic tree for the SNP cluster with the most samples (PDS000000952.271)

472 was downloaded. The common samples were marked on both the NCBI-PD and the EO phylogenetic tree
473 (Escherichia_coli_O157_H7_str_Sakai_chromosome_NC_002695_1). The marked samples on the three biggest
474 clusters on the EO tree were labeled, and their placement on the NCBI-PD tree was visually inspected.

475 Supplementary material

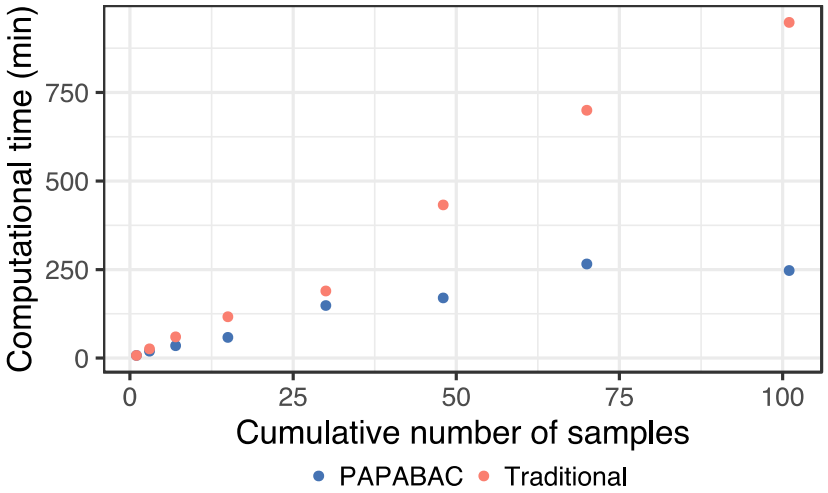
476

477 *Table S1 Non-queried species, due to mislabelled or mixed samples*

Genus	Species	Isolate
Bacillus	*subtilis*	3
Bacillus	*pumilus*	2
Campylobacter	*coli*	58
Campylobacter	*fetus*	1
Citrobacter	*amalonaticus*	1
Enterobacter	*cloacae*	2
Enterococcus	*faecalis*	1
Escherichia	*albertii*	5
Hafnia	*alvei*	3
Klebsiella	*pneumoniae*	7
Listeria	*ivanovii*	1
Morganella	*morganii*	7
Peptoclostridium	*difficile*	1
Proteus	*mirabilis*	7
Providencia	*stuartii*	2
Pseudomonas	*aeruginosa*	6
Raoultella	*ornithinolytica*	1
Salmonella	*bongori*	11
Staphylococcus	*epidermidis*	1
Streptococcus	*agalactiae*	1

478

479



480

481 *Figure S1 Computational time of the Escherichia coli in vitro evolution dataset where the samples were added in batches based on the*
482 *sampling time.*

483

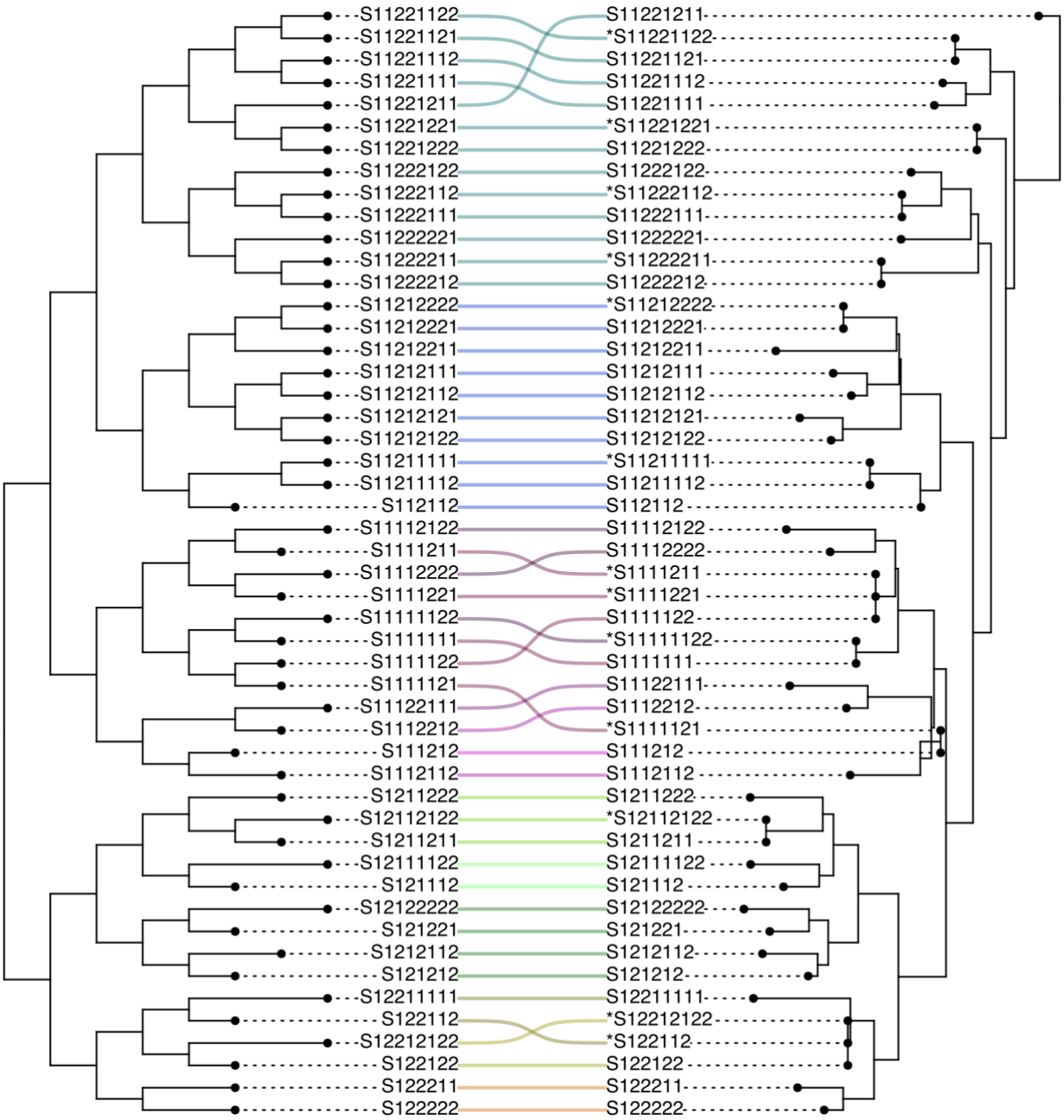


Figure S2 Comparison of the ideal tree (left) to the PAPABAC neighbor-joining tree made of the in vitro experiment dataset[31]Taxa with an asterisk were clustered together with the taxa in the same clade.

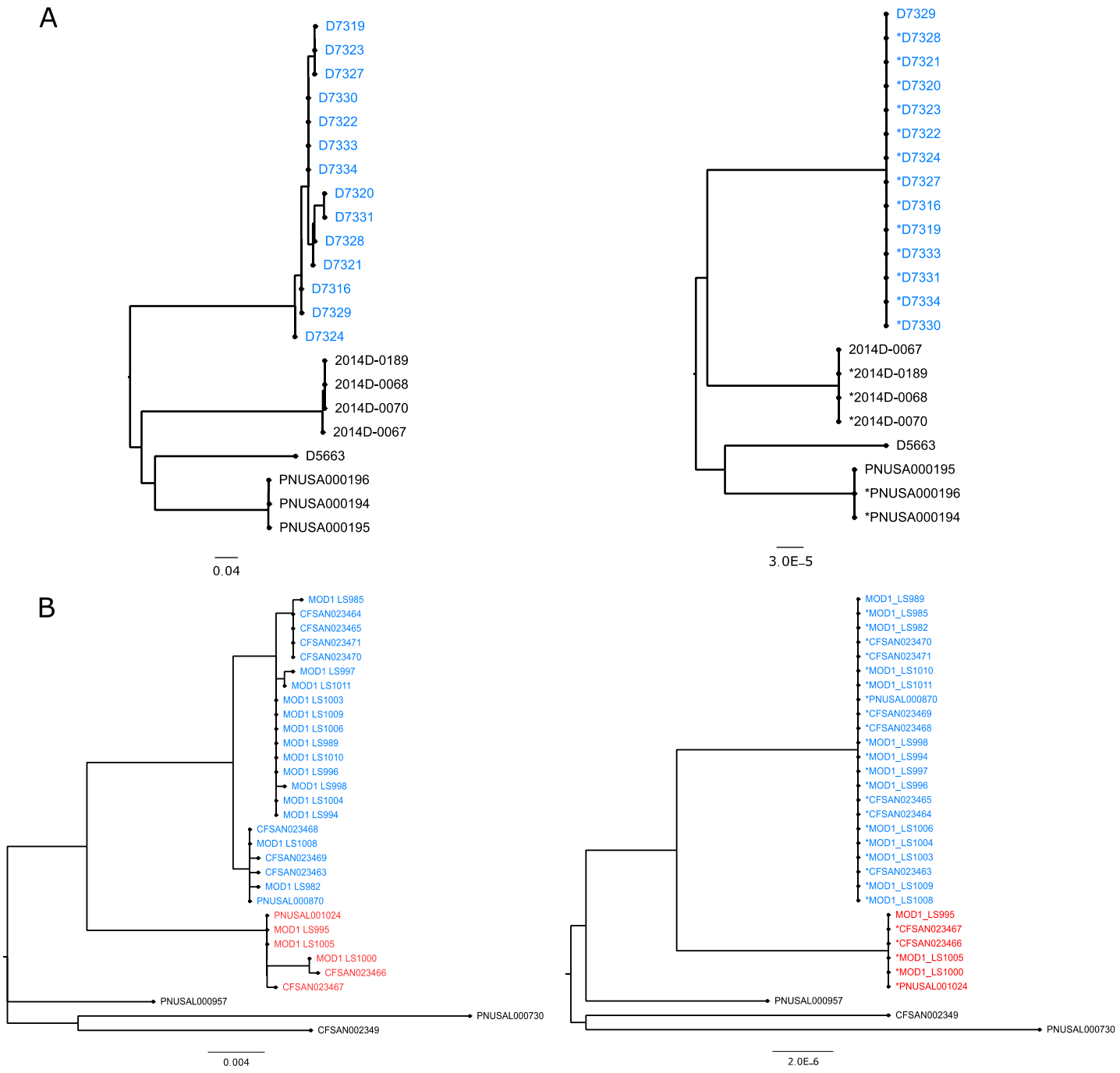


Figure S3 Maximum likelihood trees of (A) Campylobacter jejuni and (B) Listeria monocytogenes SNP pipeline benchmarking datasets. The trees on the left are the "ideal" phylogenies by Timme et al. The colored (blue, red) clades contain the outbreak strains, while the black ones are non-related isolates. The reference sequences were trimmed from the trees.

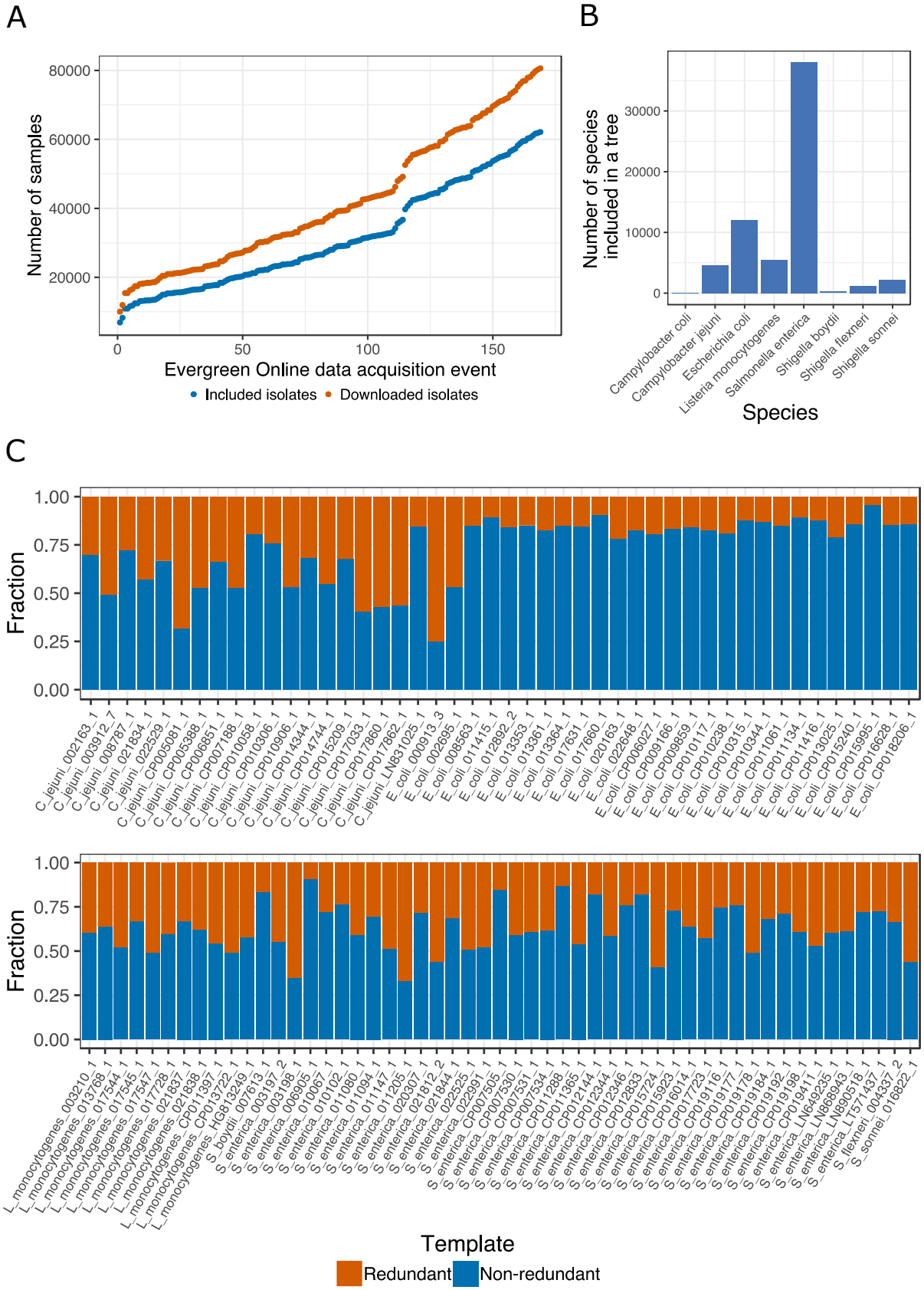


Figure S4 A) Number of downloaded and included isolates as function of data acquisition events B) Number of isolates for the species we query for C) Fraction of non-redundant isolates in template sets larger than 100 isolates