Title: A rapid and simple method for assessing and representing genome sequence relatedness.

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Abstract

Coherent genomic groups are frequently used as a proxy for bacterial species delineation through computation of overall genome relatedness indices (OGRI). Average nucleotide identity (ANI) is a widely employed method for estimating relatedness between genomic sequences. However, pairwise comparisons of genome sequences based on ANI is relatively computationally intensive and therefore precludes analyses of large datasets composed of thousand genome sequences.

In this work we evaluated an alternative OGRI based on *k*-mers counts to study prokaryotic species delimitation. A dataset containing more than 3,500 *Pseudomonas* genome sequences was successfully classified in few hours with the same precision as ANI. A new visualization method based on zoomable circle packing was employed for assessing relationships among the 350 cliques generated. Amendment of databases with these *Pseudomonas* cliques greatly improved the classification of metagenomic read sets with *k*-mers-based classifier.

The developed workflow was integrated in the user-friendly KI-S tool that is available at the following address: https://iris.angers.inra.fr/galaxypub-cfbp.

Keywords : ANI, *k*-mers, circle packing, *Pseudomonas*, metagenome

Background

Species is a unit of biological diversity. Species delineation of *Bacteria* and *Archaea* historically relies on a polyphasic approach based on a range of genotypic, phenotypic and chemo-taxonomic (e.g. fatty acid profiles) data of cultured specimens. According to the List of Prokaryotic Names with Standing in Nomenclature (LPSN), approximately 15,500 bacterial species names have been currently validated within this theoretical framework [1]. According to different estimates the number of bacterial species inhabiting planet Earth is predicted to range between 10^7 to 10^{12} species [2,3], the genomics revolution has the potential to accelerate the pace of species description.

Prokaryotic species are primarily described as cohesive genomic groups and approaches based on similarity of whole genome sequence, also known as overall genome relatedness indices (OGRI), have been proposed for delineating species. Genome Blast Distance Phylogeny (GBDP [4]) and Average nucleotide identity (ANI) are currently the most frequently used OGRI for assessing relatedness between genomic sequences. Distinct ANI algorithms such as ANI based on BLAST (ANIb [5]), ANI based on MUMmer (ANIm [6]) or ANI based on orthologous genes (OrthoANIb [7]; OrthoANIu [8]; gANI,AF [9]), which differ in their precision but more importantly in their calculation times [8], have been developed. Indeed, improvement of calculation time for whole genomic comparison of large datasets is an essential parameter. As of November 2018, the total number of prokaryotic genome sequences publicly available in the NCBI database is 170,728. Considering an average time of 1 second for calculating ANI values for one pair of genome sequence, it would take approximately 1,000 years to obtain ANI values for all pairwise comparisons.

The number of words of length k (*k*-mers) shared between read sets [10] or genomic sequences [11] is an alignment-free alternative for assessing the similarities between entities. Methods based on *k*-mer counts, such as SIMKA [10], can quickly compute pairwise comparison of multiple metagenome read sets with high accuracy. In addition, specific *k*-mer profiles are now routinely employed by multiple read classifiers for estimating the taxonomic

structure of metagenome read sets [12–14]. While these *k*-mer based classifiers differ in term of sensitivity and specificity [15], they rely on accurate genome databases for affiliating read to a taxonomic rank.

The objective of the current work was to evaluate an alternative method based on *k*-mer counts to study species delimitation on extensive genome datasets. We therefore decided to employ *k*-mer counting to assess the similarity among genome sequences belonging to the *Pseudomonas* genus. Indeed, this genus contains an important diversity of species (*n* = 207), whose taxonomic affiliation is under constant evolution [16–22], and numerous genome sequences are available in public databases. We also proposed an original visualization tool based on D3 Zoomable Circle Packing (https://gist.github.com/mbostock/7607535) for assessing relatedness of thousands of genome sequences. Finally, the benefit of taxonomic curation of reference database on the taxonomic affiliation of metagenomics read sets was assessed. The developed workflow was integrated in the user-friendly KI-S tool which is available in the galaxy toolbox of CIRM-CFBP (https://iris.angers.inra.fr/galaxypub-cfbp).

Methods

Genomic dataset

All genome sequences (*n*=3,623 as of April 2017) from the *Pseudomonas* genus were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/genome/browse#!/overview/).

Calculation of Overall Genome Relatedness Indices

The percentage of shared *k*-mers between genome sequences was calculated with Simka version 1.4 [10] with the following parameters (abundance-min 1 and *k*-mer length ranging from 10 to 20). The percentage of shared *k*-mer was compared to ANIb values calculated with PYANI version 0.2.3 (https://github.com/widdowquinn/pyani). Due to the computing time required for ANIb calculation, only a subset of *Pseudomonas* genomic sequences (*n*=934) was selected for this comparison. This subset was composed of genome sequences containing less than 150 scaffolds.

Development of KI-S tool

An integrative tool named KI-S was developed. The number of shared *k*-mers between genome sequences was initially calculated with Simka [10]. A custom R script was then employed to cluster the genome sequences according to their connected components at different selected thresholds (e.g. 50% of shared 15-mers). The clustering result is visualized with Zoomable Circle Packing representation with the D3.js JavaScript library (https://gist.github.com/mbostock/7607535). The source code of the KI-S tool is available at the following address: https://sourcesup.renater.fr/projects/ki-s/. A wrapper for accessing KI-S in a user-friendly Galaxy tool is also available at the following address: https://iris.angers.inra.fr/galaxypub-cfbp.

Taxonomic inference of metagenomic read sets

The taxonomic profiles of 9 metagenomic read sets derived from seed, germinating seeds and seedlings of common bean (*Phaseolus vulgaris* var. Flavert) were estimated with Clark version 1.2.4 [14]. These metagenomic datasets were selected because of the high relative abundance of reads affiliated to *Pseudomonas* [23]. The following Clark default parameters –k 31 –t <minFreqTarget> 0 and -o <minFreqtObject> 0 were used for the taxonomic profiling. Three distinct Clark databases were employed: (*i*) the original Clark database from NCBI/RefSeq at the species level (*ii*) the original Clark database supplemented with the 23,623 *Pseudomonas* genome sequences and their original NCBI taxonomic affiliation (*iii*) the original Clark database supplemented with the 3,623 *Pseudomonas* genome sequences whose taxonomic affiliation was corrected according to the reclassification based on the number of shared *k*-mers. For this third database, genome sequences were clustered at >50% of 15-mers.

Results

Selection of optimal *k*-mer size and percentage of shared *k*-mers

Using the percentage of shared *k*-mers as an OGRI for species delineation first required the determination of the optimal *k*-mer size. This was performed by comparing the percentage of shared *k*-mers to a widely employed OGRI, ANIb [5], among 934 *Pseudomonas* genome sequences. Since the species delineation threshold was initially proposed following the observation of a gap in the distribution of pairwise comparison values [24], the distribution profiles obtained with *k*-mer lengths ranging from 10 to 20 were compared to ANIb values. Short *k*-mers ($k < 12$) were evenly shared by most strains and not discriminative (**Fig. 1**). As the length of the *k*-mer increased, a multimodal distribution based on four peaks was observed (**Fig. 1**). The first peak related to the genome sequences that do not belong to the same species. Then, depending on *k* length, the second and third peaks (e.g. 50% and 80% for $k = 15$) corresponded to genome sequences associated to the same species and subspecies, respectively. The fourth peak at 100% of shared *k*-mers was related to identical genome sequences.

Fifty percent of 15-mers is close to ANIb value of 0.95 (**Fig. 2**), a threshold commonly employed for delineating bacterial species [5]. More precisely, the median percentage of shared 15-mers is 49% [34%-66%] for ANIb value ranging from 0.94 to 0.96. In addition, 15-mers allows the investigation of inter-and infra-specific relationship at lower and higher percentage of shared 15-mers, respectively.

Computation time of 15-mers for 934 genome sequences was 4 hours on a DELL Power Edge R510 server, while it took approximately 3 months for obtaining all ANIb pairwise comparisons (500-fold decrease of computing time).

Classification of *Pseudomonas* genome sequences

The percentage of shared 15-mers was then used to investigate relatedness between 3,623 *Pseudomonas* publicly available genome sequences. At a threshold of 50% of 15-mers, we

identified 350 cliques. The clique containing the most abundant number of genome sequences was related to *P. aeruginosa* (*n* = 2,341), followed by the phylogroups PG1 (*n* = 111), PG3 (*n* = 92) and PG2 (*n* = 74) of *P. syringae* species complex ([17]; **Table S1**). At the clustering threshold employed, 185 cliques were composed of a single genome sequences, therefore highlighting the high *Pseudomonas* strain diversity. Moreover, according to Chao1 index, *Pseudomonas* species richness is estimated at 629 cliques [± 57], which indicates that additional strain isolations and sequencing effort are needed to cover the whole diversity of this bacterial genus. Graphical representation of hierarchical clustering by dendrogram for a large dataset is generally not optimal. Here we employed Zoomable circle packing as an alternative to dendrogram for representing similarity between genome sequences (**Fig. 3** and **FigS1.html**). The different clustering thresholds that can be superimposed on the same graphical representation allow the investigation of inter- and intra- groups relationships (**Fig. 3** and **FigS1.html**). This is useful for affiliating a specific clique to a group or subgroup of *Pseudomonas* species.

Improvement of taxonomic affiliation of metagenomic read sets.

The taxonomic composition of metagenome read sets is frequently estimated with *k*-mer based classifiers. While these *k*-mer based classifiers differ in term of sensitivity and specificity, they all rely on accurate genome databases for affiliating reads to taxonomic rank. Here, we investigated the impact of database content and curation on taxonomic affiliation. Using Clark [14] as a taxonomic profiler with the original Clark database, we classified metagenome read sets derived from bean seeds, germinating seeds and seedlings [23]. Adding the 3,623 *Pseudomonas* genome sequences with their original taxonomic affiliation from NCBI to the original Clark database did not increase the percentage of classified reads (**Fig. 4**). However, adding the same genome sequences reclassified in cliques according to their percentage of shared *k*-mers (*k*=15; threshold= 50%) increased 1.4-fold on average the number of classified reads (**Fig. 4**).

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Discussion

Classification of bacterial strains on the basis on their genome sequence similarities has emerged over the last decade as an alternative to the cumbersome DNA-DNA hybridizations [4, 25]. Although ANIb is one widely employed method for investigating genomic relatedness, its intensive computational time prohibited its used for comparing large genome datasets [8]. In contrast, investigating the percentage of shared *k*-mers is scalable for comparing thousands of genome sequences.

In a method based on *k*-mer counts, choosing the length of *k* is a compromise between accuracy and speed. The distribution of shared *k*-mer values between genome sequences is impacted by *k* length. For *k* = 15, four peaks were observed at 15%, 50%, 80% and 100% of shared *k*-mers. The second peak is close to ANIb value of 0.95 and falls in the so called grey or fuzzy zone [25] where taxonomists might decide to split or merge species. Hence, according to our working dataset, it seems that 50% of 15-mers is a good proxy for estimating *Pseudomonas* clique. Despite the diverse range of habitats colonized by different *Pseudomonas* populations [20], it is likely that the percentage of shared *k*-mers has to be adapted when investigating other bacterial genera. Indeed, since population dynamics, lifestyle and location impact molecular evolution, it is somewhat illusory to define a fixed threshold for species delineation [26]. While 15-mers is a good starting point for investigating infra-specific to infra-generic relationships between genome sequences, the computational speed of KI-S offers the possibility to perform large scale genomic comparisons at different *k* sizes to select the most appropriate threshold.

Genomic relatedness using whole genome sequences has become the standard method for bacterial strain identification and bacterial taxonomy [4,25,27]. This is primarily motivated by fast and inexpensive sequencing of bacterial genomes together with the limited availability of cultured specimen for performing classical polyphasic approach. Whether full genome sequences should represent the basis of taxonomic classification is an ongoing debate between systematicians [28]. While this consideration is well beyond the objectives of

this work, obtaining a classification of bacterial genome sequences into coherent groups is of general interest. Indeed, the number of misidentified genome sequences is exponentially growing in public databases. A number of initiatives such as Digital Protologue Database (DPD [29]), Microbial Genomes Atlas (MiGA [30]), Life Identification Numbers database (LINbase [31]) or the Genome Taxonomy Database (GTDB [27]) proposed services to classify and rename bacterial strains based ANIb values or single copy marker proteins. Using the percentage of shared *k*-mers between unknown bacterial genome sequences and reference genome sequences associated to these databases could provide a rapid complementary approach for bacterial classification. Moreover, KI-S tool, provides a friendly visualization interface that could help systematicians to curate whole genome databases. Indeed, zoomable circle packing could be employed for highlighting (*i*) misidentified strains, (*ii*) bacterial taxa that possess representative type strains or (*iii*) bacterial taxa that contain few genome sequences.

Association between a taxonomic group and its distribution across a range of habitats is useful for inferring the role of this taxa on its host or environment. For instance, community profiling approaches based on molecular marker such as hypervariable regions of 16S rRNA gene have been helpful for highlighting correlations between host fitness and microbiome composition. Higher taxonomic resolution of microbiome composition could be achieved with metagenomics through *k*-mer based classification of reads. In this study we demonstrate that employing a database with a classification of strains reflecting their genomic relatedness greatly improve taxonomic assignments of reads. Therefore, investigating the relationships between bacterial genome sequences not only benefits bacterial taxonomy but also microbial ecology.

Competing interests

The authors declare that they have neither competing interests nor conflict of interest.

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Figures and Supplemental files

Figure 1: Distribution of shared *k*-mers values. Relatedness between genome sequences were estimated with ANIb (green) or shared *k*-mers (blue). The *x* axis represents ANIb or percentage of shared *k*-mers while the *y* axis represents the number of values by class in the subset of 934 *Pseudomonas* genomic comparison.

Figure 2: Comparison of various *k*-mers length and ANIb values. Pairwise similarities between genome sequences were assessed with average nucleotide identity based on BLAST (ANIb, *x*-axis) and percentage of shared *k*-mers of length 10 (**A**), 15 (**B**) and 20 (**C**). The red line corresponds to ANIb of 0.95, a threshold commonly employed for delineating species level.

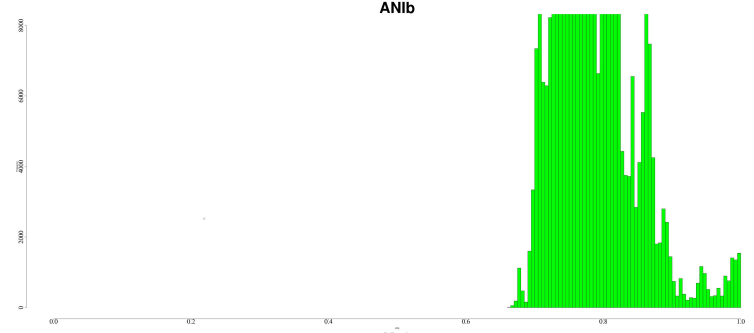
Figure 3: Hierarchical clustering of *Pseudomonas* genome sequences. Zoomable circle packing representation of *Pseudomonas* genome sequences (*n* = 3,623). Similarities between genome sequences were assessed by comparing the percentage of shared 15-mers. Each dot represents a genome sequence, which is colored according to its group of species [17,22]. These genome sequences have been grouped at three distinct thresholds for assessing infraspecific (0.75), species-specific (0.5) and interspecies relationships (0.25).

Figure 4: Percentage of classified reads. Classification of metagenome read sets derived from bean seeds, germinating seeds and seedlings with Clark [14]. Three distinct databases were employed for read classification: the original Clark database (red), Clark database supplemented with 3,623 *Pseudomonas* genome sequences (green) and the Clark database supplemented with 3,623 *Pseudomonas* genome sequences that were classified according to their percentage of shared *k*-mers (blue).

TableS1.csv : *Pseudomonas* cliques. Description of the 350 cliques obtained after clustering at 50% of shared 15-mers. For each clique, the *Pseudomonas* group [22] and subgroup [17,22] are displayed.

FigureS1.html: Zoomable circle packing representation of *Pseudomonas* genome sequences. Similarities between genome sequences were assessed by comparing the

271percentage of shared 15-mers. Each dot represents a genome sequence, which is colored
272according to its group of species [17,22]. These genome sequences have been grouped at
273three distinct thresholds for assessing infraspecific (0.75), species-specific (0.5) and
274interspecies relationships (0.25).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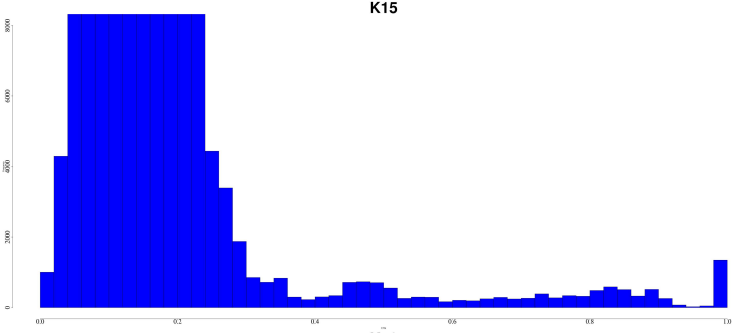
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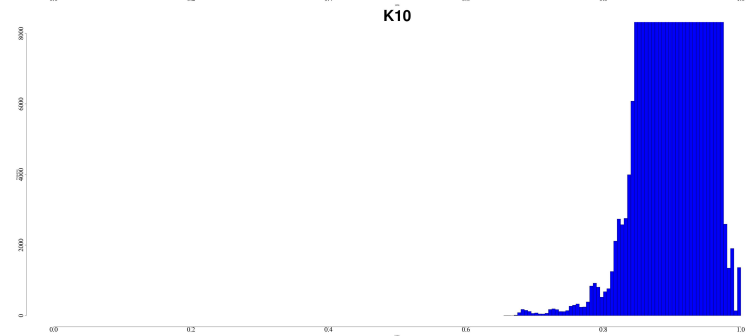
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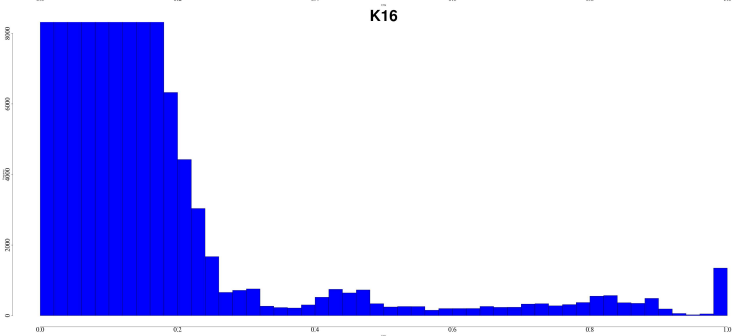
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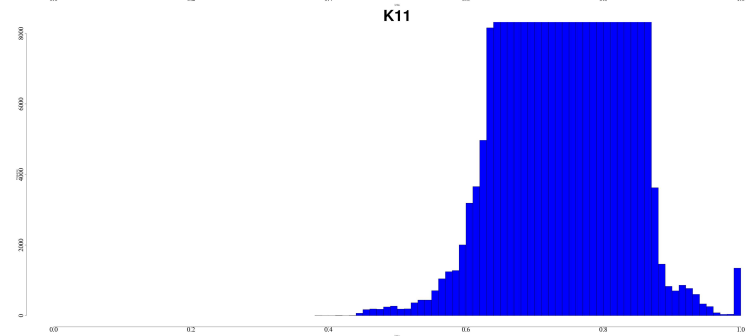
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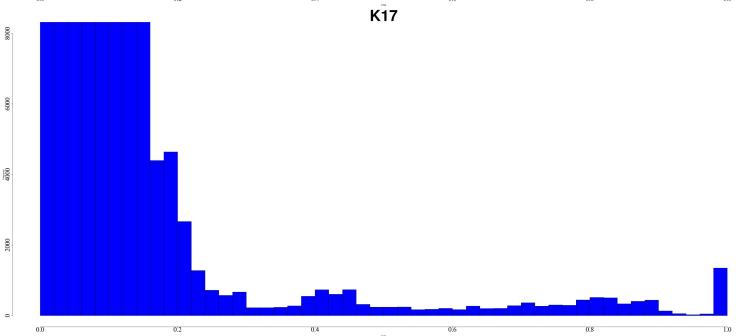
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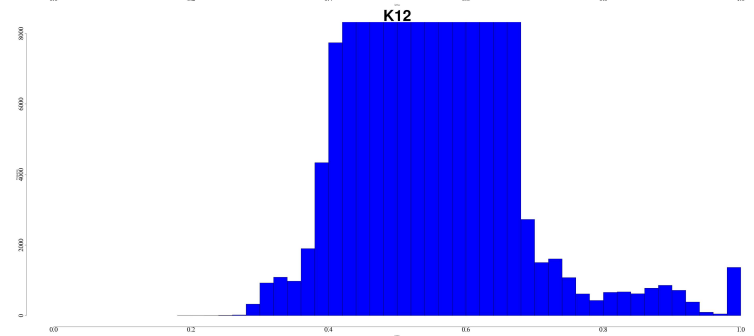
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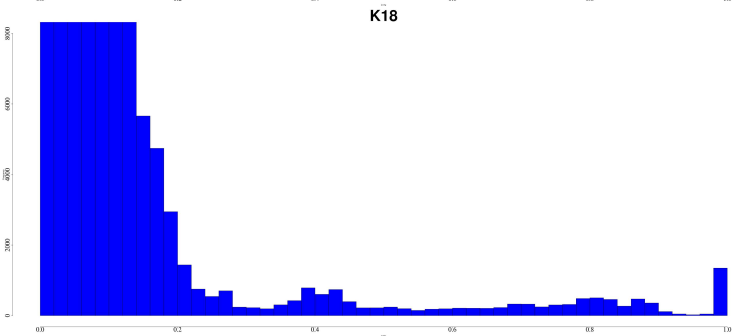
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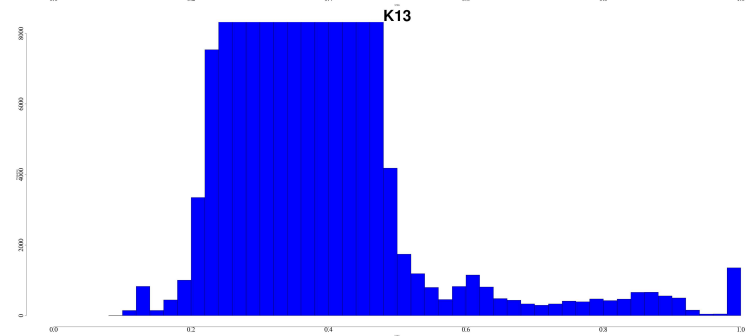
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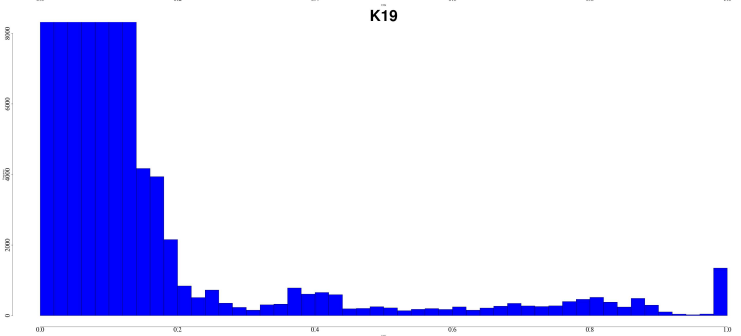
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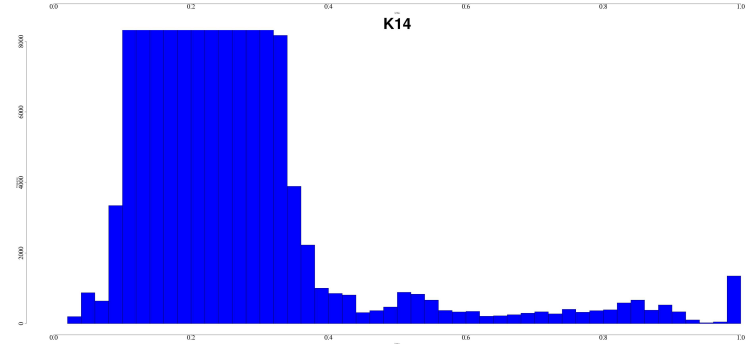
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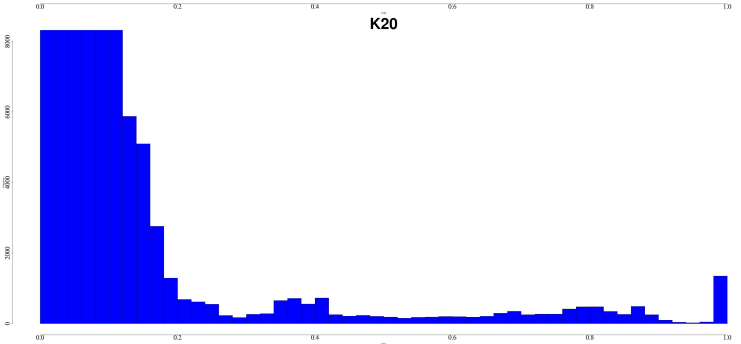
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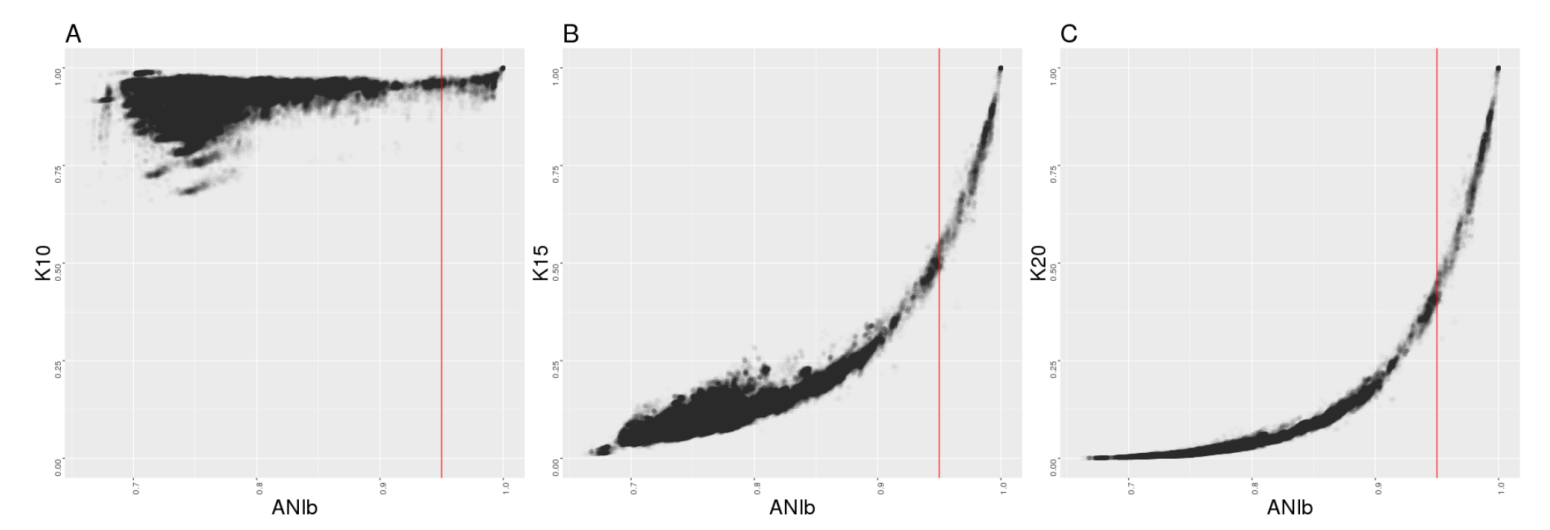
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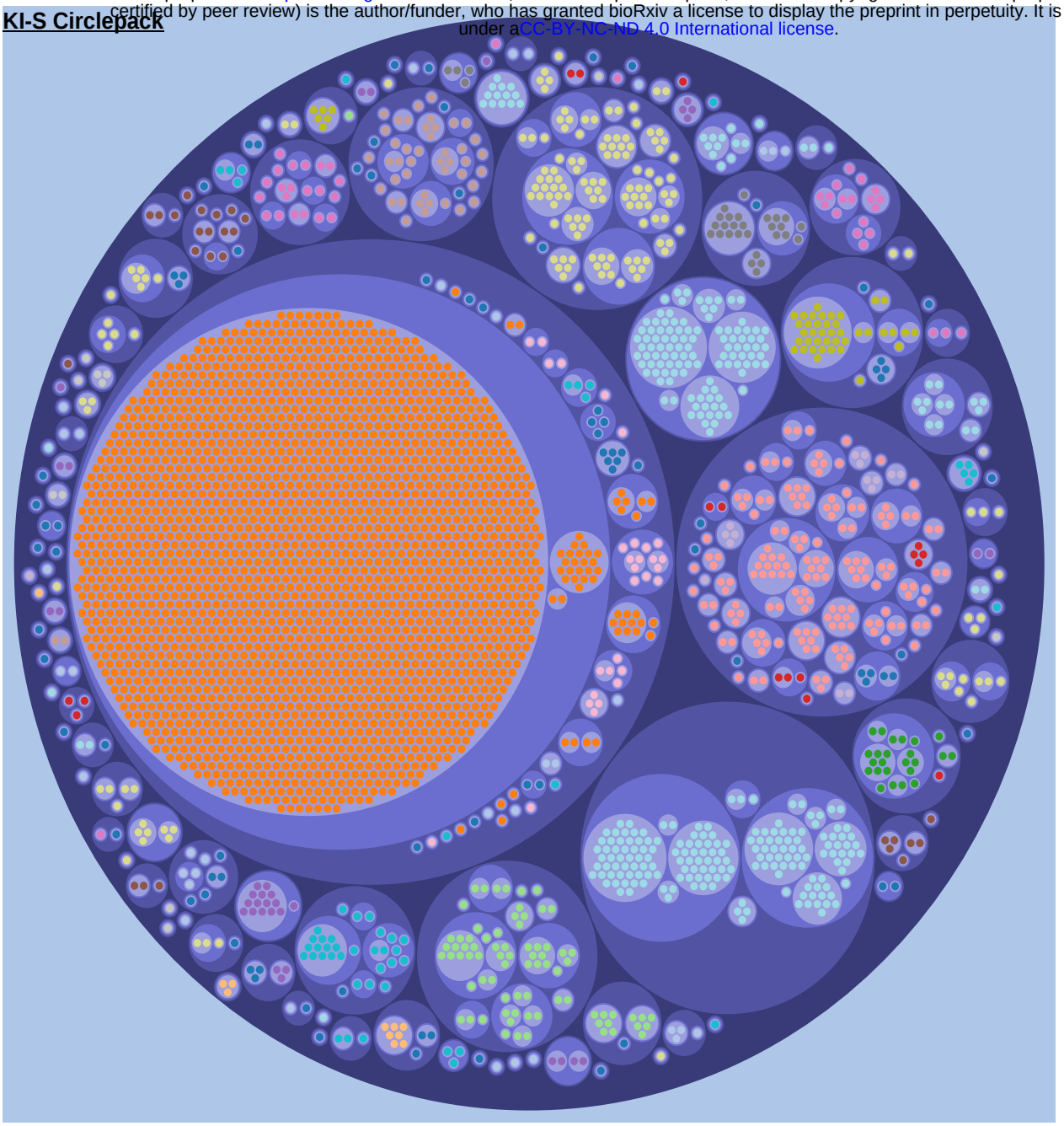
KI-S Circlepack

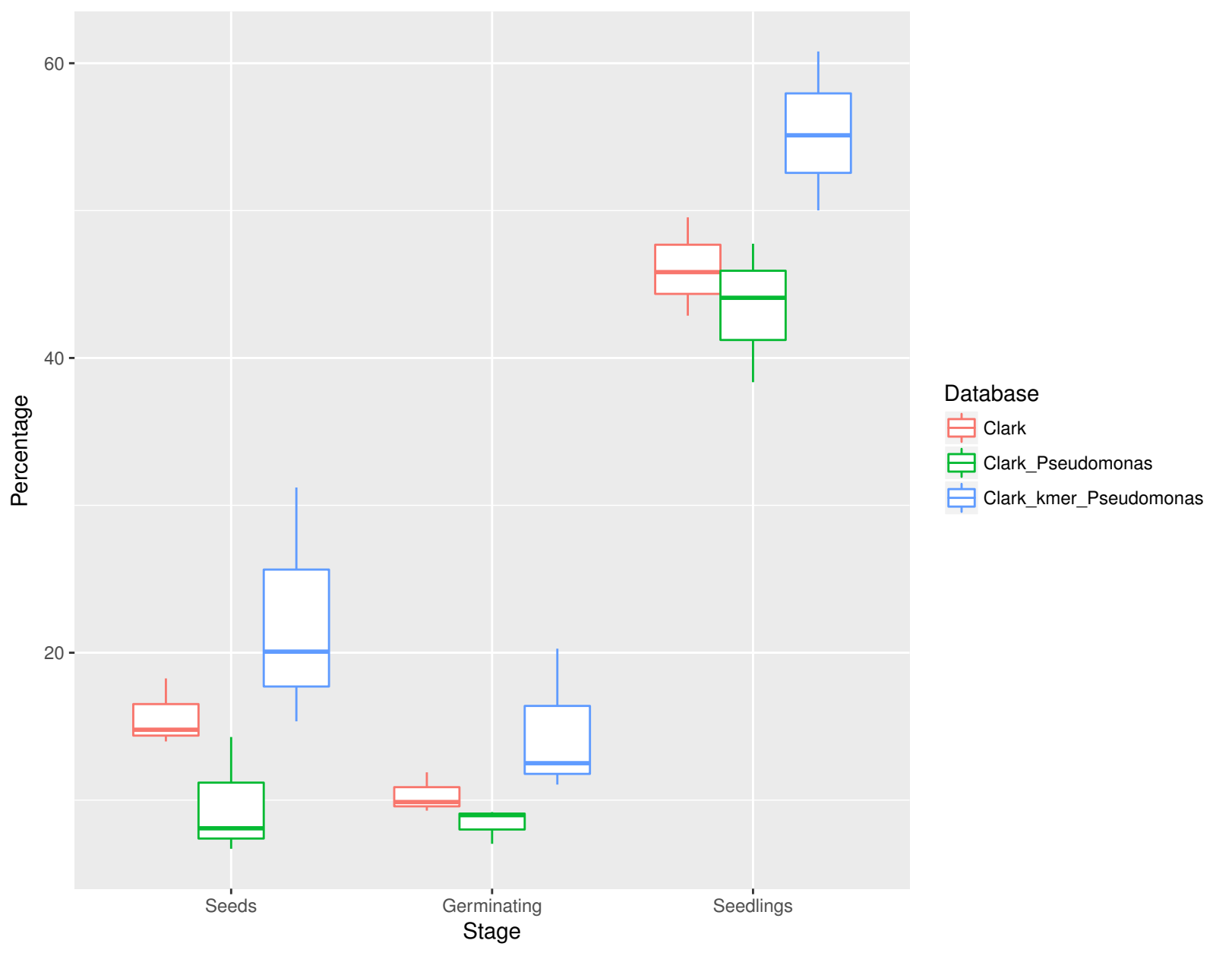


Thresholds

0.25 0.5 0.75

Groups

- P_putida_group
- P_gessardii_subgroup
- P_fragi_subgroup
- P_corrugata_group
- P_pertucinogena_group
- Other
- P_fluorescens_group
- P_aeruginosa_group
- P_jessenii_subgroup
- P_syringae_group
- P_koreensis_subgroup
- P_oryzihabitans_group
- P_protegens_subgroup
- P_stutzeri_group
- P_chlororaphis_group
- P_asplenii_subgroup
- P_fluorescens_subgroup
- P_mandelii_subgroup
- P_oleovorans_group
- Unknown


60
40
20
Percentage
Seeds
Germinating
Seedlings
Stage
Database
Clark
Clark_Pseudomonas
Clark_kmer_Pseudomonas