Spaced seeds improve metagenomic classification

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Abstract

Metagenomics is a powerful approach to study genetic content of environmental samples that has been strongly promoted by NGS technologies. To cope with massive data involved in modern metagenomic projects, recent tools [4, 39] rely on the analysis of k-mers shared between the read to be classified and sampled reference genomes. Within this general framework, we show in this work that spaced seeds provide a significant improvement of classification capacity as opposed to traditional contiguous k-mers. We support this thesis through a series a different computational experiments, including simulations of large-scale metagenomic projects.

1 Introduction

Metagenomics is a powerful approach to study genetic material contained in environmental samples. The advent of high-throughput sequencing technologies (Next-Generation Sequencing, commonly abbreviated to NGS) revolutionized this approach, by avoiding the need of cloning the DNA and thus greatly scaling up the obtention of metagenomic samples and simultaneously drastically decreasing its price. Early examples of metagenomic projects include the analysis of samples of seawater [36] [14], human gut [31], or soil [38]. Present-day metagenomic studies focus on various bacterial, fungal of viral populations, exemplified by the Human Microbiome project [30] that investigates microbial communities at different sites of human body.

Modern metagenomics deals with vast sequence datasets. On the one hand, metagenomic samples (metagenomes) obtained through NGS are commonly measured by tens or even hundreds of billions of bp [1]. These sequences generally come from a number of different species, some of which either have a previously sequenced reference genome, or have a related sequenced species sufficiently close phylogenetically to determine this relationship by sequence similarity. Other sequences, however, may come from organisms that have no sufficiently close relatives with sequenced genomes, or from DNA fragments that show no significant similarity with any available genomic sequence. The main task of metagenomic analysis, called metagenomic classification or binning, is to assign each sequence of the metagenome to a corresponding taxonomic unit, or to classify it as ‘novel’.

A way to improve the accuracy of metagenomic classification is to match the metagenome against as large set of known genomic sequences as possible. With many thousands
of completed microbial genomes available today, modern metagenomic projects match their samples against genomic databases of tens of billions of bp [39].

Alignment-based classifiers [23] proceed by aligning metagenome sequences to each of the known genomes from the predefined database, in order to use the best alignment score as an estimator of the phylogenetic “closeness” between the sequence and the genome. This could be done with generic alignment program, such as BLAST [3], BLASTZ [32], or BLAT [15]. While this approach can be envisaged for small datasets (both metagenome and database) and is actually used in such software tools as MEGAN [13] or PHYMMBL [8] (see [23] for more), it is unfeasible on the scale of modern metagenomic projects. There exists a multitude of specialized tools for aligning NGS reads – BWA [20], NOVOALIGN (http://www.novocraft.com/), GEM [25], Bowtie [18], just to mention a few popular ones – which perform alignment at a higher speed and are adjusted to specificities of NGS-produced sequences. Still, aligning multimillion read sets against thousands of genomes remains computationally difficult even with optimized tools. Furthermore, read alignment algorithms are usually designed to compute high-quality alignments only, and are often unable to report low-quality alignments. Therefore, such an approach can only be useful for screening reads with high similarity to target genomes, but not those presenting more remote similarity.

Several techniques exist to reduce the computational complexity of this approach. One direction is to pre-process the metagenomic sample in order to assemble reads into longer contigs, potentially improving the accuracy of assignment. Assembly of metagenomic reads has been subject of many works (see [34]) and remains a fragile approach, because of its error-proneness and high computational complexity. Overall, it appears feasible mostly for small-size projects with relatively high read coverage.

For large-scale metagenomic projects, alignment-based approaches appear unfeasible, and alignment-free methods are used. Alignment-free sequence comparison is in itself an established research area, reviewed in a recent dedicated special issue [37]. Most of alignment-free comparison methods are based on the analysis of words, usually of fixed size (k-mers), occurring in input sequences. A popular approach is to compute the distance between frequency vectors of all k-mers in each of the sequences. In the context of metagenomics, however, when one of the sequences is short (NGS read), the analysis is based on the shared k-mers, without taking into account their multiplicities. This is also dictated by the prohibitive computational cost of computing and storing k-mer multiplicities for metagenomics-size data.

Two recently released tools – LMAT [4] and KRaken [39] – perform metagenomic classification of NGS reads based on the analysis of shared k-mers between an input read and each genome from a pre-compiled database. Given a taxonomic tree involving the species of the database, those tools “map” each read to a node of the tree, thus reporting the most specific taxon or clade the read is associated with. Mapping is done by sliding through all k-mers occurring in the read and determining, for each of them, the genomes of the database containing the k-mer. Based on obtained counts and tree topology, algorithms [4, 39] assign the read to the tree node “best explaining” the counts.

The goal of this work is to show that the metagenomics classification based on the
analysis of shared k-mers can be improved by using spaced k-mers rather than contiguous k-mers.

The idea of using spaced k-mers goes back to the concept of spaced seeds for seed-and-extend sequence comparison [22, 10]. There, the idea is to use as a seed (i.e. local match triggering an alignment) a sequence of matches interleaved with “joker positions” holding either matches or mismatches. The pattern specifying the sequence of matches and jokers is called the spaced seed. Remarkably, using spaced seeds instead of contiguous seeds significantly improves the sensibility-selectivity trade-off without any associated computational overhead. This has been first observed in [22] and then extended and formally analysed in a series of further works, see [17, 9] and references therein.

Recently, it has been reported in several works that spaced seeds bring an improvement in alignment-free comparison as well. In [19], it is shown that comparing frequency vectors of spaced k-mers (k-mers obtained by sampling must-match positions of one or several spaced seeds), as opposed to contiguous k-mers, leads to a more accurate estimation of phylogenetic distances and, as a consequence, to a more accurate reconstruction of phylogenetic trees. In [26], the authors studied another measure – the number of pairs of matching (not necessarily aligned) spaced k-mers between the input sequences – and showed that it provides an even better estimator of the phylogenetic distance. In [28], it is shown that the number of hits of appropriately chosen spaced seeds in aligned sequences and their coverage (i.e. the total number of matched positions covered by all hits) provides a much better estimator for the alignment distance than the same measures done with contiguous seeds. From a machine learning perspective, works [29, 11] show that spaced seeds provide better string kernels for SVM-based sequence classification, confirmed by experiments with protein classification (see also [28]).

In this work, we show that using spaced k-mers is also beneficial for metagenomic classification of NGS reads, providing a higher classification accuracy. To support this thesis, we consider different scenarios. As a test case, we first study the problem of discriminating a read between two genomes, i.e. determining which of two genomes is “phylogenetically closer” to the read. We then analyse the correlation between the quality of an alignment of a read to a genome and the seed count for this read, defined either as the number of hits or as the coverage. This analysis provides an insight on how well one can estimate the similarity between a read and a genome out of k-mer occurrences. Finally, we make a series of large-scale metagenomic classification experiments with KRAKEN software [39] extended by the possibility of dealing with spaced seeds. In all our experiments we observe a superiority of spaced seeds over contiguous ones.

2 Preliminaries

A spaced seed is a binary pattern over symbols # and - denoting match and joker respectively. For example, seed #-## specifies a match followed by either match or mismatch followed by two consecutive matches. A seed acts as a mask for comparing short oligonucleotides, for example sequences gaat and gcat differ at the 2nd position, but they match via seed #-## as the 2nd position is masked out. The number of #’s in a
seed, called *weight*, defines the number $k$ of matching nucleotides. In the above example, $k = 3$ and the matching (spaced) $k$-mer is *gat*. In a slightly different terms, a seed is a pattern that specifies a small part of a gapless alignment seen as a binary sequence of matches and mismatches. For example, seed #-## occurs in (or *hits*, as usually said) any alignment containing a match followed by another two matches skipping one position to the right.

When spaced seeds are used for sequence alignment within the *seed-and-extend paradigm* \[9\], one matching $k$-mer (or, sometimes, a matching sequence of several closely located $k$-mers) indicates a potential alignment of interest in which the two $k$-mers are aligned together. When spaced seeds are used for alignment-free sequence comparison \[37\], the goal is to estimate the similarity of two sequences based on the *number* of matching $k$-mers, with no or limited information about their positions in one or both sequences. This measure can be formalized in several different ways.

In the context of metagenomic classification of NGS reads, the goal is to estimate the phylogenetic distance between a short read and a long sequence (genome), which can be modeled by the best alignment score of of the read against the sequence. Due to large genome size and large number of reference genomes involved in computations, one of the constraints is to avoid keeping track of positions of $k$-mers in genomes. We can only afford constructing an index to quickly answer queries whether a $k$-mer occurs or not in a given genome, without information on its positions. On the other hand, $k$-mer positions in the query read can be included to the analysis. Therefore, we have to derive our estimation from the number of $k$-mers shared between the read and the genome, together with their positions in the read alone.

One simple estimator is the number of $k$-mers in the read that occur in the target genome\[1\] we call this measure the *hit number*. However, one may want to favor cases when matching $k$-mers cover a larger part of the read, vs. those with matching $k$-mers clumped together due to overlaps. This leads to the concept of *coverage* \[28\], earlier used in seed-based alignment algorithms as well \[27, 7\]. The coverage is the total number of positions covered by all matching $k$-mers. For example, consider seed #-## and read *gaatcagat*. Assume the seed hits at positions 1,4,6, i.e. $k$-mers *g-at*, *t-ag* and *a-at* occur in the target genome (joker symbol is shown for the sake of clarity). Here, the hit number is 3, and the coverage is 7 as seven positions are covered by hits, namely positions 1,3,4,6,7,8,9. Hit number and coverage are two estimators studied in this work.

3 Results

3.1 Binary classification

From the machine learning viewpoint, hit number and coverage can be viewed as instances of kernel functions for sequence data (see e.g. \[6\]). Our first step was to compare their capacity w.r.t. a simple binary classification task. Assume the (impractical) case when our database contains only two genomes. Given a read, we have to decide which

\[1\]Here identical $k$-mers occurring at distinct positions in the read are considered distinct.
of the two genomes the read is closer to. How good can we be at that? Which kernel
works better for this task? And are spaced seeds better than contiguous seeds here?

3.1.1 Classifying aligned reads

As mentioned in Introduction, the “distance” of a read to a genome translates naturally
to the score of the best alignment of the read to the genome. Given two genomes, we
want to tell which of them is closer to a given read.

Consider alignments $A_s, A_l$ of a random read to the two genomes, and assume they
are gapless and have mismatch probabilities $p_s, p_l$ respectively, with $p_s < p_l$. Throughout
this paper the read length is set to 100, a typical length of ILLUMINA read. Therefore,
the alignments can be thought of as random binary strings of length 100 of matches
and mismatches, with mismatch probabilities $p_s$ and $p_l$ respectively. Given a seed, we
compute two counts $C_s$ and $C_l$ on alignments $A_s$ and $A_l$ respectively, where by ‘count’ we
mean, unless otherwise stated, either the hit number or the coverage. For example, if the
seed is #-## and the alignment 111101111 (1 stands for match and 0 for mismatch), then
the hit number is 3 and the coverage is 7. Note that in this model, common (spaced)
k-mers are assumed to occur necessarily at the same position in the read alignment,
although in reality, a k-mer of the read may not be aligned with the same k-mer in the
genome. However, in this first experiment, we abstract from this fact.

If $C_s > C_l$, then we report the correct classification; if $C_s = C_l$ then we report a
tie; otherwise we report an incorrect classification. By iterating this computation, we
estimate the probability of correct/incorrect classification for each parameter set.

The results are presented in Tables 1a, 1b, 1c.

In all cases, spaced seeds show a better classification power. In some cases, the
difference is striking: for example, if we want to discriminate between alignments with
mismatch probabilities 0.1 and 0.2 (Table 1b) using seeds of weight 22, then a spaced
seed yields 86% of correct classifications (coverage count), whereas the contiguous seed
correctly classifies only 65% of cases, whereas the fraction of incorrect classifications is
essentially the same. The results also show a slight edge of the coverage count over the
hit number, which suggests the superiority of the latter that will be confirmed later on
in other experiments.

3.1.2 Classifying unaligned reads

Let us now turn to a more practical setting, where we want to classify reads coming from
a genome $G$ between two other genomes $G_1$ and $G_2$ based on the phylogenetic closeness.

To study this, we implemented the following experimental setup. Using DWGSIM
read simulator (https://github.com/nh13/DWGSIM), we generate single-end Illumina-
like reads from genome $G$. In all experiments, we assumed 1% of base mutations (sub-
stitutions only), and 2% of sequencing errors (DWGSIM options -e 0.02 -r 0.01 -R 0).
Given a seed, (contiguous or spaced) k-mers of $G_1$ and $G_2$ are indexed to support
existence queries only. For each read, all k-mers are queried against $G_1$ and $G_2$ and
Table 1: Each entry contains a pair “Probability of correct classification/Probability of incorrect classification”. The remaining fraction estimates the probability of a tie. Spaced seeds used for hit number:

(a) \( p_s = 0.05, p_l = 0.1 \)

| weight | contiguous | spaced |
|--------|------------|--------|
|        | Hits       | Cover  | Hits   | Cover  |
| 16     | .85/.14    | .87/.11| .88/.11|
| 18     | .84/.14    | .87/.12| .88/.11|
| 20     | .83/.15    | .87/.12| .87/.12|
| 22     | .82/.16    | .86/.12| .87/.12|
| 24     | .80/.16    | .85/.12| .87/.12|

(b) \( p_s = 0.1, p_l = 0.2 \)

| weight | contiguous | spaced |
|--------|------------|--------|
|        | Hits       | Cover  | Hits   | Cover  |
| 16     | .86/.09    | .93/.05| .94/.05|
| 18     | .81/.08    | .91/.05| .92/.05|
| 20     | .74/.07    | .89/.05| .90/.05|
| 22     | .65/.06    | .85/.05| .86/.05|
| 24     | .55/.04    | .79/.04| .81/.05|

(c) \( p_s = 0.2, p_l = 0.3 \)

| weight | contiguous | spaced |
|--------|------------|--------|
|        | Hits       | Cover  | Hits   | Cover  |
| 16     | .40/.06    | .63/.07| .64/.07|
| 18     | .28/.04    | .50/.05| .50/.05|
| 20     | .18/.02    | .37/.03| .37/.03|
| 22     | .12/.01    | .25/.02| .26/.02|
| 24     | .08/.00    | .17/.01| .18/.01|

Spaced seeds used for coverage:

- `###-##-#-#---#-#-#--#--#####` (weight 16),
- `#####-###---##--#--#-#-#-##-####` (weight 18),
- `######--####----#--##-#-#--#######` (weight 20),
- `#######--####----#--##-#-#--########` (weight 22),
- `####-##-#-#-##-###-#-#--##--#####` (weight 24).
Table 2: Classification of *Mycobacterium tuberculosis* reads against *Mycobacterium marinum* and *Mycobacterium kansasii* genomes. Each entry contains a pair “Fraction (in %) of reads classified closer to *M.kansasii* / Fraction of reads classified closer to *M.marinum* ”. Spaced seeds used are 
###-#--###-##--#-#-###-#### (weight 14),
###-#--###-##--#-#-###-#### (weight 16),
###-#--###-##--#-#-###-#### (weight 18),
###-#--###-##--#-#-###-#### (weight 20),
###-#--###-##--#-#-###-#### (weight 22)
corresponding counts \( C_1 \) and \( C_2 \) are computed. If \( C_1 > C_2 \) (resp. \( C_1 < C_2 \)), the read is classified to be closer to \( G_1 \) (resp. \( G_2 \)), otherwise a tie is reported.

Note that the counts are here computed relative to the whole genome, as it is done in the approach of [4, 39] (see Introduction). This means that the \( k \)-mers occurring in the read are looked up in the whole genome, without guarantee, however, that these \( k \)-mers are closely located in the genome and contribute to the same read alignment. This makes the seed weight an important parameter, as seeds of low weight may result in a high read count which does not evidence any alignment of the read due to random character of the \( k \)-mer hits.

We experimented with bacterial genomes belonging to *Mycobacterium* genus. Members of this genus present low interspecies genetic variability and their phylogeny remains uncertain [35].

If \( G \) coincides with one of \( G_1, G_2 \), i.e. reads have to be classified between its source genome and a related genome, then all estimators correctly classify nearly all reads as soon as \( G_1 \) and \( G_2 \) are genomes of distinct species. For example, classifying reads obtained from *Mycobacterium tuberculosis* against *M. tuberculosis* itself and *M. avium* led to more than 99% of correct classifications for both spaced and contiguous seeds of all weights between 14 and 22, with the exception of contiguous seed of weight 22 which classified 95% only.

The case when \( G \) is distinct from \( G_1, G_2 \) appears more interesting. It corresponds to the real-life metagenomic setting where reads to be classified can come from a genome that is not represented in the database. Here we expect our procedure to determine whether \( G \) is phylogenetically closer to \( G_1 \) or to \( G_2 \).

For example, we classified *M.tuberculosis* reads against *M.kansasii* and *M.marinum* genomes. Results, shown in Table 2, support that *M.tuberculosis* is closer to *M.kansasii* than to *M.marinum*. The distinction is somewhat erratic for weight 14 but gets more consistent for larger weights. This result invalidates the phylogenies shown in Fig.1 and Fig.2 of [35], while supporting the phylogenies of Fig.3 and Fig.4 of [35].

|                  | weight 14 | weight 16 | weight 18 | weight 20 | weight 22 |
|------------------|-----------|-----------|-----------|-----------|-----------|
| contig hit nb    | 51 / 42   | 43 / 35   | 28 / 23   | 18 / 14   | 12 / 09   |
| contig cover     | 48 / 48   | 47 / 39   | 29 / 23   | 18 / 14   | 12 / 09   |
| spaced hit nb    | 49 / 39   | 45 / 38   | 31 / 24   | 25 / 19   | 18 / 13   |
| spaced cover     | 51 / 45   | 47 / 40   | 33 / 25   | 26 / 19   | 19 / 13   |
As another example, we classified *M. vanbaalenii* reads against *M. smegmatis* and *M. gilvum* genomes. Similar to the previous case, alternative phylogenies of [35, Fig.1-4] imply different evolutionary scenarios for these three species. Our results, shown in Table 3, suggest that *M. vanbaalenii* is closer to *M. smegmatis* than to *M. gilvum*. Importantly, this conclusion is supported by seeds of weight 16 or more, while weight 14 supports the opposite conclusion. This is due to spurious hits that become dominating when the weight drops to 14, and to the larger size of *M. smegmatis* genome (6.99Mbp) compared to *M. gilvum* (5.62Mbp). This example confirms that seeds of weight smaller than 16 should not be used for classification against whole genomes.

We also performed a series of experiments with the large and genetically variable *Bacillus* genus. Table 4 shows a demonstrative experiment with members of *B. cereus* group: *B. thuringiensis* (serovar konkukian str.), *B. anthracis* (Ames str.), and *B. cereus* (ATCC 14579 str.). The three bacteria are close to the point of being considered to be different lineages of a single *B. cereus* species [12]. The results provide a strong support that the *B. thuringiensis* strain is closer to the *B. anthracis* strain than to the *B. cereus* strain, which agrees with phylogenies reported in the literature [2]. Indeed, the *B. thuringiensis* strain and the *B. anthracis* strain have a much higher pairwise identity rate than the former has with the *B. cereus* strain (estimated DNA-DNA hybridization distance 81% vs. 45%, as computed by GGDC [5]).

However, for distinct species with low sequence similarity, a large majority of reads
Table 5: Classification of *Bacillus licheniformis* reads against *Bacillus anthracis* and *Bacillus pumilus* genomes. Each entry contains a pair “Fraction (in %) of reads classified closer to *B*. *anthracis* / Fraction of reads classified closer to *B*. *pumilus*”. Spaced seeds used are shown in Table 2.

### Weight 18

|               | 14  | 16  | 18  | 20  | 22  |
|---------------|-----|-----|-----|-----|-----|
| contig hit nb | 47 / 40 | 24 / 25 | 04 / 07 | 01 / 04 | 00 / 02 |
| contig cover  | 49 / 46 | 24 / 25 | 04 / 07 | 01 / 04 | 00 / 02 |
| spaced hit nb | 50 / 30 | 23 / 25 | 04 / 08 | 01 / 07 | 01 / 03 |
| spaced cover  | 55 / 37 | 23 / 25 | 04 / 08 | 01 / 07 | 01 / 03 |

may have no hits to either genome, and only a small fraction of reads may reveal a significant difference in distance. This situation is illustrated in Table 5 showing the results of classification of *B. licheniformis* (ATCC 14580 str.) reads against *B. anthracis* (Ames str.) and *B. pumilus* genomes (SAFR 032 str.). Starting from seed weight 18, the results support a higher similarity of *B. licheniformis* to *B. pumilus* than to *B. anthracis*, but the difference is revealed on a very small fraction of reads. The conclusion, however, is significant as this small fraction of reads faithfully reflects the identity level in similarity regions between genomes. As with the previous example, this result is confirmed by previously reported phylogenies [2].

In all our experiments reported in this section, spaced seeds showed a significantly better classification capacity. This especially applies to “nontrivial” cases involving relatively dissimilar genomes, such as those illustrated by Tables 2, 3 and 5. On the other hand, the difference between hit number and coverage estimators appeared insignificant, in accordance with results of Section 3.1.1.

### 3.2 Correlation of counts with alignment quality

In metagenomic projects, reads to be classified do not necessarily come from genomes stored in the database, but can come from genomes of other species. These species can be genetic variants of species of the database, such as different strains of the same bacteria, but can also come from organisms represented in the database only at the rank of genus or family, or may even have no representatives at all at low taxonomic ranks. Therefore, an accurate mapping of a read to a corresponding clade requires not just assigning it to the appropriate sampled genome, but estimating its distances to each of the genomes in order to locate its position within the whole taxonomic tree.

With this motivation, we turned to the question how well the measured counts correlate with the alignment score. For a fixed minimal identity rate \( p_{id} \), we randomly sampled gapless alignments of length 100 with identity rate from interval \( [p_{id} - .1, 1] \), and collected pairs (number of mismatches, count), where, as before, ‘count’ stands for either the number of hits or the coverage of a given seed. For these data, we computed Spearman’s rank correlation.

The results are presented on plots in Figure 1. They show that when the identity...
Figure 1: Spearman’s rank correlation between score and counts, depending on the minimal identity rate and on whether the seed is contiguous or spaced
rate of alignments takes a large range of values (minimal id rate smaller than \( \approx 0.9 \)), spaced seeds yield a significantly higher correlation than contiguous seeds, for both hit-number and coverage counts. Furthermore, the coverage count slightly outperforms the hit-number count, especially for spaced seeds and larger weights.

For high-similarity alignments, however, the picture changes: the coverage count loses its performance, with its correlation value sharply decreasing. Furthermore, the correlation of hit-number goes down as well for spaced seeds, while it continues to grow for contiguous seeds ending up by reaching and even slightly outperforming the one for spaced seed. These phenomena are due to combinatorial reasons: when the number of mismatches is small, the number of hits of a contiguous seed correlates very well with the number of mismatches, compared to a spaced seed. This is due to a larger span of spaced seeds and to their combinatorial properties that cause the hit number values to be less sharply concentrated at certain values, and therefore to be less well correlated with the number of mismatches.

In conclusion, while spaced seeds provide a much better estimator for alignments whose quality ranges over a large interval, for high-quality alignments (> 90% of identity), the hit number of contiguous seed becomes a better estimator. The superiority of hit-number over coverage for high-quality alignments has also been reported in [28]. Along with Spearman’s correlation, we also made an analysis of mutual information computed on the same data (data not shown) that confirmed the above conclusions.

### 3.3 Correlation on real genomes

To validate the conclusions of the previous section in a real-life metagenomics framework and to analyse more closely how well different counts for a read correlate with the best alignment of the read to a real genomic sequence, we implemented another series of experiments.

Given a genome \( G \), we generated a set of Illumina-like single-end reads by selecting random substrings of \( G \) of length \( L \) (\( L = 100 \)) and introducing \( k \) mismatch errors, with \( k \) drawn randomly between 1 and 20 for every read. For each read, we computed the counts – hit number and coverage – with respect to genome \( G \) under a given seed, similar to Section 3.1.2. Collected data have then been analysed.

This experimental setup has been applied to *Mycobacterium tuberculosis* genome, a typical result (seed weight 20) is shown in Figure 2. Each plot shows the density of reads for each pair (number of mismatches, count), depending on the seed (contiguous or spaced) and count (hit-number or coverage). Spearman’s and Pearson’s correlation coefficients are shown for each plotted dataset.

The plots clearly illustrate the advantage of spaced seeds over contiguous seeds for estimating the alignment quality. Plots for contiguous seeds are more blurred whereas plots for spaced seeds demonstrate a better correlation between the two values. This is confirmed by the absolute values of Spearman’s rank correlation coefficient that are significantly higher for spaced seeds, indicating a better statistical dependence. This is further illustrated in Figure 3 which shows the same data through the average curve and
Figure 2: Hit number (left plots) and coverage (right plots) depending on the number of mismatches in randomly generated reads. Seed is shown above the plot, and Spearman’s and Pearson’s correlations are shown below. Grayscale shows the density of reads. Experiments made on *M. tuberculosis* genome.
95% confidence band. It confirms that spaced seeds produce a dependence with lower deviation from the mean, compared to contiguous seeds.

Comparing hit-number and coverage estimators, we observe that coverage yields a slightly better Spearman correlation and a significantly better Pearson correlation, due to a convex shape of the dependence, compared to the more straight dependence for the coverage.

This analysis has been done for several other bacterial genomes, producing similar results. Plots for other genomes and other seed weights can be found at https://github.com/gregorykucherov/spaced-seeds-for-metagenomics.

3.4 Large-scale experiments: plugging spaced seeds into Kraken software

In order to validate the advantage of spaced seeds in large-scale metagenomic projects, we modified Kraken software [39] to make it work with spaced seeds rather than with contiguous seeds only. The limitation of this comparison is that it only allows estimating the effect of using spaced seeds combined with the Kraken algorithm, and within its implementation. On the other hand, this procedure allows us to estimate the effect of spaced seeds in an unbiased manner, by keeping unchanged all other factors that might influence the results.

Our extended implementation, that we call seed-Kraken, allows the user to specify a spaced seed as a parameter. For a set of genomes, a database of spaced $k$-mers matching the seed is constructed, which is later used to classify reads through the original Kraken algorithm. Since Kraken uses $k$-mer counting Jellyfish program [24] as the $k$-mer indexing engine, we had also to modify Jellyfish to allow it to deal with spaced $k$-mers.

Integration of spaced seed into Kraken required a minor modification of the way Kraken deals with complementary sequences. In Kraken, complementary $k$-mers have a single representative in the index, the lexicographical smallest of the two. With spaced seeds, dealing with complementary sequencing is more delicate, as the complement of a spaced $k$-mer does not match the same seed but its inverse. To cope with this, we modified Kraken to index each distinct $k$-mer. We then processed each read in direct and complementary directions separately and select the one which produced more hits. Compared to original Kraken, this procedure takes more index space and doubles $k$-mer query time, but provides in return a better precision as it avoids mixing up hits coming from complementary strands.

To compare the performance of Kraken and seed-Kraken, we classified 10,000 sequences from each of three simulated metagenomes HiSeq, MiSeq, and simBA-5 introduced in the original work [39]. HiSeq and MiSeq metagenomes are based on whole-genome Illumina shotgun reads of 20 bacterial genomes, while simBA-5 metagenome is a set of simulated replicons from 607 genera with error rate five fold of the one expected in real experiments. Due to resource limitations, the database we used in the experiment was half of the size of the Kraken’s default database (which requires 70GB of RAM). Our database was constructed by choosing from each bacteria species a single representative strain, except for the species from HiSeq and MiSeq metagenomes for which all
Figure 3: Same data as in Figure 2 shown with averages and 95% confidence intervals
Figure 4: Performance of classification of seed-KRAKEN (spaced seed modification), and original KRAKEN, on three simulated metagenomes HiSeq (10 bacterial genomes, low error rate), MiSeq (10 bacterial genomes, average error rate), simBA-5 (607 bacterial genera, high error rate). Charted are genus precision (positive predictive value) against genus sensitivity (rate of correct assignments). Varying are k-mer length, and its spaced seed equivalent seed weight, while the seed span is kept constant at 31. seed-KRAKEN consistently outperforms original KRAKEN in sensitivity/sensibility trade off.
strains were included. Thus, the results on HiSeq and MiSeq datasets are similar to Kraken’s original experiment, while simBA-5 result differs in reported sensitivity.

In Figure 4, sensitivity and precision at the genus level are charted for seed-Kraken, and for the unmodified Kraken. By varying spaced seed weight, an equivalent parameter to k-mer length in original Kraken, we observe the Receiver Operating Characteristic (ROC) of seed-Kraken to be above its unmodified counterpart, by up to 2 percentage points, and this result is consistent in all three simulated metagenomes. This confirms spaced seeds superiority in optimizing sensitivity/sensibility trade off. Yet in seed-Kraken there is another natural parameter which can be varied, namely the span of the spaced seed. For this experiment we set the span to 31, as well as k-mer length in Kraken, as it is the case that current implementation of Kraken is limited to k-mers of maximal length 31.

Building a seed-Kraken database takes approximately 1 hour on a server with 20 CPU cores, and the resulting size was 52 GB for seed of weight 24, which is comparable to the original. However, a separate database has to be built for each spaced seed analysed, e.g. each different seed span or weight. The classification running times are bigger than for original Kraken by a factor of 3 to 5 reaching approximately 70 Mbp/min.

4 Conclusions

Through a series of computational experiments, we showed that spaced seeds significantly improve the accuracy of metagenomic classification of short NGS reads. The superiority of spaced seeds for different variants of alignment-free sequence comparison has been recently demonstrated by other authors as well [29, 11, 19, 28]. In this work, we specifically focused on the metagenomics setting, characterized by very large volumes of data, both in terms of the number of reads and the size of genomic database. This quantity of data precludes using some alignment-free comparison techniques, and leaves room only for highly time- and space-efficient approaches. Note also that in our setting, we have to compare short sequences (reads) with long ones (whole genomes), which makes an important difference with problems considered in [19, 28]. For example, in the framework of metagenomic classification, it is hardly conceivable taking into account k-mer frequencies [19], as this information would be computationally difficult to utilize.

Another enhancement considered in [19, 28] is to use multiple seeds, i.e. several seeds simultaneously instead of a single one. This extension is known to bring an advantage in seed-and-extend sequence alignment [33, 21], and [19, 28] show that this improvement applies to alignment-free comparison as well. However, each seed requires to build a separate index for database genomic sequences, and therefore it appears computationally difficult to use multiple seeds in metagenomics, unless some new indexing techniques are designed for this purpose.

In our work, we studied two estimators: hit number and coverage. We also experimented with another measure defined as the number of non-overlapping hits (taken left-to-right). While showing good characteristics in many situations, this measure is in general not “fine-grained” enough to be used a distance estimator. One may, however,
think of other measures such as a weighted number of hits where each hit is given a weight depending on, e.g., its overlap with other hits or some external knowledge such as the rate of sequencing error in this part of the read. This provides an issue for further study.

We now summarize the main contributions of our work.

- We showed that spaced seeds can drastically improve the success rate of binary classification of alignments into two categories, each defined by a specific mismatch rate. Here the classification is done through “querying” an alignment using a seed as a mask and reporting whether the seed applies at a given position. For example, in discriminating between alignments of length 100 with mismatch rate 0.2 and 0.3, a spaced seed of weight 16 achieves 63% of success while a contiguous seed of the same weight achieves only 40% (Table 1c).

Recently, spaced seeds have been shown to define more efficient kernels for SVM classification of both protein [29] and nucleotide (RNA) sequences [28]. Compared to these works, here we demonstrate the superiority of spaced seeds in a very simple classification setting, where sequences have to be classified according to identity rate, without a training stage and without resorting to SVM machinery.

- Through examples on bacterial genomes of *Mycobacterium* genus, we showed experimentally that spaced seeds allow for a better classification of NGS reads coming from a genome *G* between two other genomes *G*₁ and *G*₂ of the same genus. Here reads are classified according to the phylogenetic distance between *G* and *G*₁ and *G* and *G*₂ respectively. We established that correct classification is obtained with seeds of weight 16 and more, while seeds of weight 14 are insufficient. We also observed that coverage measure provides an insignificant advantage over hit-number in this task.

- We studied how well different estimators (coverage/hit-number combined with spaced/contiguous seed) correlate with the alignment quality, by measuring Spearman’s rank correlation coefficient and mutual information coefficient. Here again, we observed a significantly better correlation produced by spaced seeds, but only when the alignment quality varies over a sufficiently large range, starting from identity rate around 0.9 or below. On the other hand, if only high-quality alignment are targeted (id rate at least 0.9) then the correlation produced by spaced seeds becomes lower, with hit-number measure over a contiguous seed eventually becoming the best for alignments of id rate about 0.95 or more.

- We also measured the correlation produced on real genomes within the metagenomic classification approach of [4, 39]. For this, we assumed that the “closeness” of a read to a genome is characterized by the quality of the alignment (in our case, the number of mismatches), and computed the correlation produced by different counts on simulated reads. These experiments confirmed the superiority of spaced seeds as well. Moreover, they showed that coverage combined with spaced seeds provides the best option, leading to the highest Spearman’s correlation but also
to a significantly higher Pearson’s correlation. The latter means that this estimator induces a dependency closer to linear, which can be a useful feature for classification algorithms.

- Finally, we compared spaced and contiguous seeds through large-scale metagenomics experiments. We modified Kraken software \cite{kraken} to make it work with spaced seeds, without modifying the core classification algorithm or any other parts of the software, with the only exception being the way the complementary sequences are dealt with. With just replacing contiguous seeds by spaced seeds, Kraken showed a significant improvement of specificity/sensitivity trade-off.

All our experiments corroborate the thesis of better performance of spaced seeds for metagenomic classification. Many different questions are raised by this work. Our results remain to be explained with more rigorous probabilistic arguments, similarly to how it has been done for spaced seeds applied to seed-and-extend paradigm \cite{skamene1991}. While there are obvious similarities between the two applications, the underlying “mechanisms” seem to be different. One sign of this difference is that optimal spaced seeds for the two problems are not the same, i.e. seeds maximizing the hit probability are not those maximizing the correlation of counts. Furthermore, seeds optimized for hit-number and coverage counts are generally not the same \cite{hashimoto2019}. In our experiments, we used seeds maximizing Pearson’s correlation between the coverage or hit-number and the real distance. Seed design has been done with IEDERA software (http://bioinfo.lifl.fr/yass/iedera) \cite{iedera} recently extended for this purpose \cite{hashimoto2019}. Real data experiments of Sections 3.1.2, 3.3 have been done using SnakeMake \cite{snakemake}.

Experiments with Kraken (Section 3.4) give a strong evidence that spaced seeds can improve the classification accuracy in real-life large-scale metagenomic projects. One further improvement would be to implement the coverage measure, as it showed, in general, a better performance compared to the hit number. Introducing spaced seeds rise new issues, such as the construction of an efficient index of the database, or adapting the algorithm of computing the most likely node of the taxonomic tree from counts produced by individual genomes, i.e. leaves of the tree. These questions are subject of our future work.

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