## SKraken: Fast and Sensitive Classification of Short Metagenomic Reads based on Filtering Uninformative k-mers

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#### Keywords: Metagenomic Reads Classification, Representative k-mers.

Abstract: The study of microbial communities is an emerging field that is revolutionizing many disciplines from ecology to medicine. The major problem when analyzing a metagenomic sample is to taxonomic annotate its reads in order to identify the species in the sample and their relative abundance. Many tools have been developed in the recent years, however the performance in terms of precision and speed are not always adequate for these very large datasets. In this work we present SKraken an efficient approach to accurately classify metagenomic reads against a set of reference genomes, e.g. the NCBI/RefSeq database. SKraken is based on *k*-mers statistics combined with the taxonomic tree. Given a set of target genomes SKraken is able to detect the most representative *k*-mers for each species, filtering out uninformative *k*-mers. The classification performance on several synthetic and real metagenomics datasets shows that SKraken achieves in most cases the best performances in terms of precision and recall w.r.t. Kraken. In particular, at species level classification, the estimation of the abundance ratios improves by 6% and the precision by 8%. This behavior is confirmed also on a real stool metagenomic sample where SKraken is able to detect species with high precision. Because of the efficient filtering of uninformative *k*-mers, SKraken requires less RAM and it is faster than Kraken, one of the fastest tool.

Availability: https://bitbucket.org/marchiori\_dev/skraken

#### **1 INTRODUCTION**

Metagenomics is the study of genomic sequences in a heterogeneous microbial sample (e.g. soil, water, human microbiome) (Mande et al., 2012; Felczykowska et al., 2012). One of the primary goals of metagenomic studies is to determine the taxonomical identity of the microorganisms that are present in a sample.

Several research areas, investigating various microbiomes, have been revolutionized by this emerging field like ecology, medicine, microbiology, and other (Consortium, 2012; Qin et al., 2010; Zeller et al., 2014; Said et al., 2014). For the first time researchers are now able to study the genomic material in environmental samples without the need to separate and culture bacteria or of biased preprocessing steps. This analysis can reveal the presence of unexpected bacteria and viruses in a microbial sample, and it also allows the identification and characterization of bacterial and viral genomes at a level of detail not previously possible. For example, in the case of the human body, imbalances in the microbiome are related with many diseases, e.g. inflammatory bowel disease (IBD) (Qin et al., 2010) and colorectal cancer (Zeller et al., 2014).

The taxonomic classification of metagenomics reads is in general performed with two techniques: (1) sequencing phylogenetic marker genes, e.g. 16S rRNA; (2) NGS sequencing of all the genomic material in the sample. The use of marker genes requires amplification steps that can introduce bias in the taxonomic analysis. Moreover, not all bacteria can be identified by traditional 16S sequencing, because of its divergent gene sequences (Brown et al., 2015).

The most effective and unbiased method to study microbial samples is via high-throughput sequencing. However, the short length of NGS reads poses a number challenges for the correct taxonomical classification of each read. Several methods and software tools are available, but with the increasing throughput of modern sequencing technologies faster and more accurate algorithms are needed. These methods can be broadly divided into three categories: (1) sequence

In Proceedings of the 10th International Joint Conference on Biomedical Engineering Systems and Technologies (BIOSTEC 2017) - Volume 3: BIOINFORMATICS, pages 59-67 ISBN: 978-989-758-214-1

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DOI: 10.5220/0006150500590067

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similarity based methods, (2) marker-based methods where certain specific marker sequences are used to identify the species. (3) sequence composition methods, which are based on the nucleotide composition (e.g. k-mers usage).

The sequence similarity based methods search reads in reference databases through sequence similarity, popular example are MegaBlast (Zhang et al., 2004) and Megan (Huson et al., 2007). They are very precise at identifying reads from genomes within the reference database, but they are generally very slow, especially compared with composition based methods. Marker-based methods try to mimic the taxonomic classification of marker genes (Liu et al., 2011; Caporaso et al., 2010; Segata et al., 2012). For example, MetaPhIAn (Segata et al., 2012) is based on marker genes that are clade specific.

The fastest and most promising approaches are based on sequence composition. In these methods, first the genomes of reference organisms are modeled based on *k*-mers counts, then reads are searched into this reduced database and classified based on the origin of the hit sequences. On this category the most representative methods are Kraken (Wood and Salzberg, 2014), Clark (Ounit et al., 2015) and Lmat (Ames et al., 2013). The performance of these methods in terms of precision are as good as MegaBlast (Zhang et al., 2004), but they are much faster. Thus, only these methods are really capable to keep pace with the increasing throughput of modern sequencing instruments.

A recent comparison of different metagenomic classification methods has shown that Kraken (Wood and Salzberg, 2014) is one of the most promising tool in terms of both similarity to the correct answer and classification speed (Lindgreen et al., 2016). The key idea behind the success of Kraken is the construction of a database of all reference genomes based on the *k*-mers of each genome combined with a taxonomic tree. More precisely, Kraken constructs a data structure that is an augmented taxonomic tree in which a list of significant *k*-mers is associated to each node, leafs as well as internal nodes. Given a node on this taxonomic tree, its list of *k*-mers is considered representative for the taxonomic label of the node and used for the classification of metagenomic reads.

Inspired by this paradigm, in this paper we propose SKraken a tool for metagenomics reads classification that selects the most representative k-mers for each node in the taxonomic tree, filtering out uninformative k-mers. The main properties of SKraken can be summarized as follows: i) an efficient detection of representative k-mers over the taxonomic tree; ii) SKraken improve the precision of Kraken on sim-

ulated and real metagenomic datasets without compromising the recall. iii) The database of reference genomes constructed by SKraken is 8% smaller than the one computed by Kraken. As a consequence, SKraken requires less memory RAM and the classification speed increases w.r.t. Kraken, one of the fastest tool. In the next section we give an overview of Kraken and analyze how to improve the classification. SKraken is presented in section 2.1. Both tools are tested on simulated and real metagenomic datasets in section 3 and the conclusions are drawn in section 4.

#### 2 METHODS

In order to better understand our contribution here we briefly describe Kraken. One the major novelties of Kraken is the ability to efficiently index a large database of target genomes, e.g. all the genomes in RefSeq. Instead to use the complete genome as reference Kraken, as well as many other tools (Ounit et al., 2015; Ames et al., 2013), considers only its *k*-mers, thus a genome is represented by its constituent k-mers.

This relatively simple idea has profound implications, and it is at the base of alignment-free methods (Vinga and Almeida, 2003). Researchers have shown that the use of k-mers frequencies for comparing sequences has proved useful in different applications. The construction of phylogenetic trees, traditionally based on a multiple-sequence alignment, can be carried out on whole genomes (Sims et al., 2009; Comin and Verzotto, 2012). Several k-mers based methods have been devised for the detection of enhancers in ChIP-Seq data (Goke et al., 2012; Kantorovitz et al., 2007; Comin and Verzotto, 2014; Antonello and Comin, 2015) and also of entropic profiles (Antonello and Comin, 2013; Antonello and Comin, 2014). The assembly-free comparison of genomes and metagenomes based on NGS reads and k-mers counts has been investigated only recently (Comin and Schimd, 2014; Comin et al., 2015; Schimd and Comin, 2016; Ondov et al., 2016). For a comprehensive review of alignment-free measures and applications we refer the reader to (Vinga and Almeida, 2003).

The basic idea of Kraken is to consider the taxonomic tree, taken from the complete NCBI taxonomic information, and extend this data structure by annotating each node, leafs as well as internal nodes, with k-mers. At every node is associated a list of k-mers that are considered important for that node and that will be used for classification. Given a dataset of target genomes, the construction of this annotated tax-



Example of Taxonomic Annotation of K-mer

AGCCT AGCCT Figure 1: In this example the *k*-mer AGCCT, that is contained in the species 9 and 13, is moved to the lowest common

onomic tree is carried out by scanning the *k*-mers of each genome in the dataset. If the *k*-mer appears only in a given genome, than it is associated to the leaf representing the species of that genome and the list of *k*-mers of the node is updated. If the *k*-mer appears in more than one species then its moved to the lowest common ancestor of these nodes, see Figure 1 for an example. At the end of this step each *k*-mer is associated with only one node in the taxonomic tree.

ancestor, the family node 2.



Figure 2: An overview of the metagenomic reads classification of Kraken (Wood and Salzberg, 2014).

Once this database of annotated k-mers has been constructed, Kraken can classify reads in a very efficient manner. Figure 2 reports an overview of the classification process. Given a read Kraken decomposes the read into a list of its k-mers. Then each kmer is searched in the augmented taxonomic tree, and in the corresponding node a counter is incremented for every hit. Once all k-mers have been analyzed the node's counters and the taxonomic tree are used to classify the read by searching the highest weighted path, from the root to a leaf, in the tree.

### 2.1 SKraken: Selecting Informative *k*-mers

The most important step of Kraken is the construction of the augmented taxonomic tree. In this data structure k-mers are associated with nodes and then used for the taxonomic classification of reads. In this paper we propose SKraken that follows a similar paradigm for the classification step. The major differences are in the construction of the augmented taxonomic tree, where uninformative k-mers are carefully selected and filtered.

One of the problem with the augmented taxonomic tree of Kraken is that it is enough that two species share a k-mer to insert this k-mer is the list of important features of an internal node. If we consider the example in Figure 1, we can note that the k-mer AGCCT is moved from the leaf nodes 9 and 13, representing two species, to the node 2 representing a taxonomic family. Since this k-mer will be used in the classification step, we would like to be informative for the family node 2. However, the majority of species in this family, nodes 10, 11 and 12, do not contain this k-mer.

To address this issue, for each k-mer, we define a scoring function that captures its representativeness with respect to a taxonomic node. We recall that a k-mer is associated with only one node in the tree.



Figure 3: An example of quality score q(GAACT).

Let's define TaxID(m) as the taxonomic node associated with the k-mer m. However, the k-mer m can occur in many difference species, leaf nodes. We define NumSpecies(m) as the the number of species that contains m. By construction TaxID(m) is the lowest common ancestor of all these species. Thus the species in which m appears, they are all leafs node of the subtree rooted in TaxID(m). We define TotSpecies(n) as the total number of species in the subtree routed in the node n. With these values we define q(m) the quality of a k-mer m as :

# $q(m) = \frac{NumSpecies(m)}{TotSpecies(TaxID(m))}$

Figure 3 shows an example of the quality q(GAACT). The quality of GAACT can also be interpreted as the percentage of species nodes that contains GAACT, i.e. NumSpecies(GAACT), with respect to the family node 2, i.e. TaxID(GAACT), in this case 60%. Similarly, if we consider the example in Figure 1, the quality of q(AGCCT) = $\frac{NumSpecies(AGCCT)}{TotSpecies(TaxID(AGCCT))} = \frac{2}{5} = 0.4$ , that is 40%. Thus, if a k-mer has an high quality can be considered representative for a given taxonomic node, and the related subtree, and more likely will be informative for the classification. Based on these observations SKraken selects uninformative k-mers, by means of their quality, and it prunes the augmented taxonomic tree by removing the k-mers with a quality below a given threshold Q.

In order to compute the quality scores q(m) for all *k*-mers we need to be able to evaluate efficiently *NumSpecies*(*m*) and *TotSpecies*(*n*). The construction of the augmented taxonomic tree of SKraken is divided into two steps. In the first step, given a set of target genomes, we scan the k-mers of each genome and build the augmented taxonomic tree, similarly to Kraken. In addition, in this phase, for each k-mer *m* we keep a variable to compute NumSpecies(m). Every time *m* is found in a new species we increment this variable. However, there can be genomes that are further classified as sub-species of a given species node. In order to compute the correct value of NumSpecies(m), we need to make sure that all genomes of a given species are processed before moving to next species. This can be obtained by scanning the input genomes in a particular order so that all genomes of a species, and eventually sub-species, are processed at once. Another problem is the fact a k-mer can appears in many sub-species of a given species node. When computing NumSpecies(m) we need to make sure not to overcount these occurrences, and thus the corresponding variable is incremented only when m is found for the first time in a given species. All other occurrences of m within the same species will be discarded. At the end of the first phase we have computed the augmented taxonomic tree, with all k-mers, and the corresponding values NumSpecies(m).

In the second phase SKraken computes the quality values q(m) and filters uninformative k-mers. The number of leaf nodes descendants of n, TotSpecies(n), can be obtained for all nodes in the tree with a post-order traversal of the taxonomic tree. Then all k-mers are processed and the corresponding qualities q(m) are computed. If q(m) is below a given input parameter Q, m is removed from the database.

Note that the size of the taxonomic tree is con-

Туре	Dataset	Reads	Species	Reads Length
Single-end	HiSeq	10000	10	92
Single-end	MiSeq	10000	10	100
Single-end	simBA5	10000	1216	100
Paired-end	Mix1	1000000	10	100
Paired-end	Mix2	1000000	10	100
Paired-end	SRR1804065	1053741	775	100

Table 1: A summary of simulated and real metagenomics datasets.

stant and much smaller with respect to the number of k-mers. The overall process depends only on the total number of k-mers and it is linear in size of the input reference genomes. Once the augmented taxonomic tree is build reads can be classified with the same procedure of Kraken.

#### **3 RESULTS**

The comparison of different metagenomic classification methods is a non-trivial task (Lindgreen et al., 2016). Since our algorithm is inspired by Kraken, one of the best performing methods (Lindgreen et al., 2016), we compare SKraken with it as reference tool. These tools require to build a reference database, we use as input for both all bacterial and archaeal complete genomes in NCBI RefSeq. In order to test the performance of SKraken we used several metagenomic datasets representing simulated and real communities. The simulated and real datasets are obtained from the original paper of Kraken (Wood and Salzberg, 2014) as well as from other related studies (Girotto et al., 2016; Ounit et al., 2015)). The simulated datasets represent five mock communities that are constructed from real sequencing data: MiSeq, HiSeq, Mix1, Mix2, simBA5. The MiSeq and HiSeq metagenomes were built using 10 sets of bacterial whole-genome shotgun reads. Mix1 and Mix2 are based on the same species of HiSeq, but with two different abundance profiles.

The MiSeq dataset is particularly difficult to analyze because it contains five genomes from the *Enterobacteriaceae* family (*Citrobacter, Enterobacter, Klebsiella, Proteus and Salmonella*). The high sequence similarity of this family can make the classification of the MiSeq dataset more difficult. The metagenome simBA5 was created by simulating reads from the complete set of bacterial and archaeal genomes in RefSeq, for a total of 1216 species. It contains reads with an high error rate and it was created with the purpose to evaluate the performance on datasets with many errors and many species.

We also evaluated the performance of SKraken on

a real stool metagenomic sample (SRR1804065) from the Human Microbiome Project. Because there is no ground truth for this dataset, we use BLAST to find the reads that uniquely map, with a sequence identity of 95%, to a genome and filter out all other reads. If two paired-end reads do not map on the same genome we discard them. As a result the real metagenomic sample contains 775 distinct species and 1053741 reads. A summary of the main characteristics of all simulated and real metagenomics datasets can be found in Table 1.

In order to compare the results we used the standard metrics of precision and recall. Given N the number of reads, Y the number of reads classified and X the number of reads correctly classified, we define precision as the fraction of correct assignments over the total number of assignments (X/Y), and recall as the ratio between the number of correct assignments and the number of reads to be classified (X/N). If one is interested in the number of reads that remains unclassified, it can be indirectly estimated from the recall. In fact the percentage of reads unclassified is bounded above by 1 - recall. When analyzing a metagenomic sample one need to verify that the abundance ratios of species estimated by the tools is similar to the known profile. To test also this important aspect we compute the Pearson correlation between the estimated abundance profile and the known ratios. A Pearson correlation of 0 means that the distribution of abundance ratios are very different, whereas a correlation of 1 that they match perfectly the correct abundance profile.

For Kraken we use the default parameter k = 31 because, as suggest by the authors (Wood and Salzberg, 2014), it is the best balance between precision and recall. For SKraken we use the same value of k = 31 and we test the performance varying the filtering parameter Q.

To assess the performance of SKraken we devised a series of tests varying the parameter Q and the taxonomic level at which the classification is evaluated. In the first set of experiments we want to test how the filtering parameter Q impact the performance metrics. We run Kraken and SKraken on the dataset Mix1 and evaluate the classification accuracy at the species-



Species-level Classification with Various Filters



level. The results are reported in Figure 4. If the parameter Q = 0 all k-mers are kept and there is not filtering, thus the performance of Kraken and SKraken are identical. As Q grows we can see that the precision improves from 63% to 75%, whereas the recall remains constant. However, it is not obvious that by classifying more reads correctly also the distribution of species is consistent with the correct profile. For this reason, another important observation is that also the Pearson correlation with the known abundance ratios also increases. This behavior is observed also for the other datasets (data not shown).

Thus, we use the most stringent filtering (Q = 100%) to classify all dataset at the species-level. Figure 5 shows a summary of precision and recall for all simulated and real metagenomic datasets. This test confirms that SKraken is able to improve the precision on all datasets without compromising the recall. On simulated metagenomes the average precision increases on from 73% of Kraken to 81% of SKraken. Also on the real metagenome, where the performance of Kraken are excellent with a precision of 91%, SKraken achieves 96%.



Figure 5: Precision and Recall of Species-level classification of Kraken and SKraken (Q = 100%) for all datasets.

In general the study of metagenomic sample re-

quires an analysis in depth of the genomic content, and for this reason researchers focus at the lowest taxonomic level, species. However metagenomic reads can be mapped at a higher level, thus the classification at the genus-level is also of interest. We performed a set of experiments similar to the ones above, considering the genus taxonomic level for classification. At first we try to use as filtering parameter Q = 100%, and the results are in Figure 6. If we observe the performance of Kraken at genus level we can see that are better than those at species level, as expected. In fact, in the taxonomy tree, when the classification level is more specific, the label assignment is more difficult. Moreover, it is possible that, although at species level a read is assigned a wrong label, at genus level the same label is indeed correct, thus making genus level classification relatively less difficult. In fact the average precision of Kraken is 96% at genus-level and 73% at species-level.

SKraken 100% - Genus-level Classification



Figure 6: Precision and Recall of Genus-level classification of Kraken and SKraken (Q = 100%) for all datasets.

With filtering parameter Q = 100% the precision improves in almost all datasets, however the recall of SKraken decreases. If we consider a less stringent threshold Q = 25% (see Figure 7), we can obtain results that are in line with the previous experiments, with a moderate improvement in the precision and recall almost unchanged. A possible explanation of the small gain in terms of precision is the fact that the classification at the genus level is relatively easier, and Kraken has already very good performance.





Figure 7: Precision and Recall of Genus-level classification of Kraken and SKraken (Q = 25%) for all datasets.

In the last series of experiments we test the ability to detect the correct abundance ratios in a metagenomic sample. The Pearson correlation is used to compare the estimated abundances with the known ones. Figure 8 reports the correlations for various level of classification. The correlations of Kraken at the genus level are very high and difficult to improve, however SKraken obtains similar results on almost all metagenomes with the exception of simBA5 where the correlation increases from 0.92 to 0.97. This dataset, simBA5, is also one of the most complex and realistic metagenomes with 1216 species. If we compare these Pearson correlations with those of species level classification in general the values decrease confirming that it is more difficult to detect the correct species, rather than the genus. This is the case where the classification accuracy can benefit from a careful selection of discriminative k-mers. In fact for all dataset the correlation of SKraken is better the original algorithm. The average Pearson correlation of Kraken is 0.71 and of SKraken is 0.77. Again, in one of the most difficult metagenome, simBA5, the improvement is substantial from 0.61 to 0.77.

To summarize the results of these experiments on different simulated and real datasets have shown that SKraken is able to improve the precision in the taxonomic classification of metagenomic reads without compromising the recall. Not only more reads are classified to the correct taxa, but also the estimated abundance ratios is better than the one computed by Kraken. An important property of SKraken is that the impact on these metrics improves as the taxonomic level evaluated in the classification becomes



Figure 8: The Pearson correlation of the estimated abundances with the correct ratios for various level of classification and parameters.

lower and thus more difficult. Moreover, as the number of newly sequenced species grows the probability that two non-related species share a given k-mer will grows. For this reason we conjecture that SKraken will be able to remove more uninformative k-mers as the number of sequenced genomes increases.

#### 3.1 Filtered *k*-mers and Memory

Another effect of SKraken is that, since k-mers are filtered, the size of the augmented taxonomic tree decreases. This database needs to be loaded in memory in full during the classification step. The size of the database produced by Kraken, when using all bacterial and archaeal complete genomes in NCBI RefSeq, is about 65GB and it contains 5.8 billion k-mers.



Figure 9: Percentage of k-mers filtered and Database size as a function of the quality threshold Q.

In Figure 9 we evaluate the percentage of k-mers filtered and the impact in memory for different values of threshold Q. As expected, the percentage of k-mers filtered grows with the threshold Q and it reaches the maximum of 8.1% with Q = 100. By construction, the impact in memory depends linearly by the number of k-mers to be indexed. When using the most stringent filtering, Q = 100, SKraken requires to index 5.3 billion k-mers in 60GB of space. This reduction decreases the amount of RAM required for classification, but also it increases correspondingly the classification speed.

#### 4 CONCLUSIONS

The taxonomic classification of metagenomics reads remains a crucial step in many metagenomics analysis. In this work we presented SKraken an approach based on filtering uninformative k-mers. We compared the classification performances of SKraken on several synthetic and real metagenomics datasets, showing that SKraken achieves in most cases the best performances in terms of precision and recall w.r.t. Kraken. In particular the precision at species level classification improves by 8%. In the estimation of the abundance ratios in a metagenomic sample SKraken obtains good results on all datasets. This behavior is confirmed also on a real stool metagenomic sample where SKraken is able to detect species with high precision. Another desirable property is that SKraken requires less amount of RAM w.r.t. Kraken. As future direction of investigation it would be interesting to explore alternative definitions of k-mer quality incorporating other topological information of the tree of life.

#### ACKNOWLEDGEMENTS

The authors would like to thank the anonymous reviewers for their valuable comments and suggestions. This work was supported by the Italian MIUR project PRIN20122F87B2.

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