KrakenHLL: Confident and fast metagenomics classification using unique k-mer counts

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Abstract

False positive identifications are a significant problem in metagenomic classification. We present KrakenHLL, a novel metagenomic classifier that combines the fast k-mer based classification of Kraken with an efficient algorithm for assessing the coverage of unique k-mers found in each species in a dataset. On various test datasets, KrakenHLL gives better recall and F1-scores than other methods, and effectively classifies and distinguishes pathogens with low abundance from false positives in infectious disease samples. By using the probabilistic cardinality estimator HyperLogLog (HLL), KrakenHLL is as fast as Kraken and requires little additional memory.

Keywords: metagenomics, microbiome, metagenomics classification, pathogen detection, infectious disease diagnosis

Background

Metagenomic classifiers attempt to assign a taxonomic identity to each read in a data set. Because metagenomics data often contain tens of millions of reads, classification is typically

done using exact matching of short words of length *k* (*k*-mers) rather than alignment, which would be unacceptably slow. The results contain read classifications but not their aligned positions in the genomes [as reviewed by 1]. However, read counts can be deceiving. Sequence contamination of the samples–introduced from laboratory kits or the environment during sample extraction, handling or sequencing–can yield high numbers of spurious identifications [2, 3]. Having only small amounts of input material can further compound the problem of contamination. When using sequencing for clinical diagnosis of infectious diseases, for example, less than 0.1% of the DNA may derive from microbes of interest [4, 5]. Additional spurious matches can result from low-complexity regions of genomes and from contamination in the database genomes themselves [6].

Such false-positive reads typically match only small portions of a genome; e.g., if a species' genome contains a low-complexity region, and the only reads matching that species fall in this region, then the species was probably not present in the sample. Reads from microbes that are truly present should distribute relatively uniformly across the genome rather than being concentrated in one or a few locations. Genome alignment can reveal this information. However, alignment is resource intensive, requiring the construction of indexes for every genome and a relatively slow alignment step to compare all reads against those indexes. Some metagenomics methods do use coverage information to improve mapping or quantification accuracy, but these methods require results from much slower alignment methods as input [7]. Assembly-based methods also help to avoid false positives, but these are useful only for highly abundant species [8].

Here, we present KrakenHLL, a novel method that combines very fast k-mer based classification with a fast k-mer cardinality estimation. KrakenHLL is based on the Kraken metagenomics classifier [9], to which it adds a method for counting the number of unique k-mers identified for each taxon using the efficient cardinality estimation algorithm HyperLogLog [10-12]. By counting how many of each genome's unique k-mers are covered by reads, KrakenHLL can often discern false positive from true positive matches. Furthermore, KrakenHLL implements additional new features to improve metagenomics classification: (a) searches can be done against multiple databases hierarchically, (b) the taxonomy can be extended to include nodes for strains and plasmids, thus enabling their detection, and (c) the database build script allows the addition of >100,000 viruses from the NCBI Viral Genome Resource [13]. KrakenHLL provides a superset of the information provided by Kraken while running equally fast or slightly faster, and while using very little additional memory during classification.

Results

KrakenHLL was developed to provide efficient k-mer count information for all taxa identified in a metagenomics experiment. The main workflow is as follows: As reads are processed, each k-mer is assigned a taxon from the database (Figure 1A). KrakenHLL instantiates a HyperLogLog data sketch for each taxon, and adds the k-mers to it (Figure 1B and Supplementary Information). After classification of a read, KrakenHLL traverses up the taxonomic tree and merges the estimators of each taxon with its parent. In its classification report, KrakenHLL includes the number of unique k-mers and the depth of k-mer coverage for each taxon that it observed in the input data (Figure 1C).

[FIGURE 1]

Figure 1. Overview of the KrakenHLL algorithm and output. (A) An input read is shown as a long gray rectangle, with k-mers shown as shorter rectangles below it. The taxon mappings for each k-mer are compared to the database, shown as larger rectangles on the right. For each taxon, a unique k-mer counter is instantiated, and the observed k-mers (K7, K8, and K9) are added to the counters. (B) Unique k-mer counting is implemented with the probabilistic estimation method HyperLogLog (HLL) using 16KB of memory per counter, which limits the error in the cardinality estimate to 1% (see main text). (C) The output includes the number of reads, unique k-mers, duplicity (average time each k-mer has been seen) and coverage for each taxon observed in the input data.

Efficient k-mer cardinality estimation using the HyperLogLog algorithm

Cardinality is the number of elements in a set without duplicates; *e. g.* the number of distinct words in a text. An exact count can be kept by storing the elements in a sorted list or linear probing hash table, but that requires memory proportional to the number of unique elements. When an accurate estimate of the cardinality is sufficient, however, the computation can be done efficiently with very small amount of fixed memory. The HyperLogLog algorithm (HLL) [10], which is well suited for k-mer counting [14], keeps a summary or *sketch* of the data that is sufficient for precise estimation of the cardinality, but requires only a small amount of constant space to estimate cardinalities up to billions. The method centers on the idea that long runs of leading zeros, which can be efficiently computed using machine instructions, are unlikely in random bitstrings. For example, about every fourth bitstring in a random series should start with 01_2 (one 0-bit before the first 1-bit), and about every 32nd hash starts with 00001_2. Conversely, if we know the maximum number of leading zeros k of the members of a random set, we can use

93 2^{k+1} as a crude estimate of its cardinality (more details in the Suppl. Methods). HLL keeps $m=2^p$
94 one-byte counts of the maximum numbers of leading zeros on the data (its data *sketch*), with p,
95 the precision parameter, typically between 10 and 18 (see Figure 2). For cardinalities up to $m/4$,
96 we use the sparse representation of the registers suggested by Heule et al. [11] that has the much
97 higher effective precision p' of 25 by encoding each index and count in a vector of four-byte
98 values. To add a k-mer to its taxon's sketch, the k-mer (with k up to 31) is first mapped by a hash
99 function to a 64-bit hash value. Note that k-mers that contain non-A, C, G or T characters (such
100 as ambiguous IUPAC characters) are ignored by KrakenHLL. The first p bits of the hash value
101 are used as index i, and the later $64-p=q$ bits for counting the number of leading zeros k. The
102 value of the register $M[i]$ in the sketch is updated if k is larger than the current value of $M[i]$.
103
104 When the read classification is finished, the taxon sketches are aggregated up the taxonomy tree
105 by taking the maximum of each register value. The resulting sketches are the same as if the k-
106 mers were counted at their whole lineage from the beginning. KrakenHLL then computes
107 cardinality estimates using the formula proposed by Ertl [12], which has theoretical and practical
108 advantages and does not require empirical bias correction factors [10, 11]. In our tests it
109 performed better than Flajolet's and Heule's methods (Suppl. Figures 1 and 2).
110
111 The expected relative error of the final cardinality estimate is approximately $1.04/\mathrm{sqrt}(2^p)$ [10].
112 With $p=14$, the sketch uses 2^{14} one-byte registers, i.e. 16 KB of space, and gives estimates with
113 relative errors of less than 1% (Figure 2). An exact counter would require about 40 MB per
114 million distinct k-mers when implemented using an unordered set; i. e. about 40 GB for the
115 pathogen identification samples with an average of one billion distinct k-mers per sample.

116 However, unordered sets have worst case insertion time complexity linear to the container size

117 (and require re-hashes on resize), while it is constant for HLL.

118

[FIGURE 2]

p	$m=2^p$	Space (kB)	Rel. Error
10	1024	1	3.25%
11	2048	2	2.23%
12	4096	4	1.63%
13	8192	8	1.15%
14	16384	16	0.81%
15	32768	32	0.57%
16	65536	64	0.41%
17	131072	128	0.29%
18	262144	256	0.20%
25			0.02%

119 Figure 2: Cardinality estimation using HyperLogLog for randomly sampled k-mers from

120 microbial genomes. Left: standard deviations of the relative errors of the estimate with precision

121 p ranging from 10 to 18. No systematic biases are apparent, and, as expected, the errors decrease

122 with higher values of p. Up to cardinalities of about $2^p/4$, the relative error is near zero. At higher

123 cardinalities, the error boundaries stay near constant. Right: the size of the registers, space

124 requirement, and expected relative error for HyperLogLog cardinality estimates with different

125 values of p. For example, with a precision p=14, the expected relative error is 0.81% and the

126 counter only requires 16 KB of space, which is three orders of magnitude less than that of an

127 exact counter (at a cardinality of one million). Up to cardinalities of $2^p/4$, KrakenHLL uses a

128 sparse representation of the counter with a higher precision of 25 and an effective relative error

129 rate of about 0.02%.

130

131 **Results on twenty-one simulated and ten biological test datasets**

132 We assessed KrakenHLL's performance on the 34 datasets compiled by McIntyre et al. [15] (see
133 Suppl. Table 3). We place greater emphasis on the eleven biological datasets, which contain
134 more realistic laboratory and environmental contamination. In the first part of this section, we
135 show that unique k-mer counts provide higher classification accuracy than read counts, and in
136 the second part we compare KrakenHLL with the results of eleven metagenomics classifiers. We
137 ran KrakenHLL on three databases: 'orig', the database used by McIntyre et al., 'std', which
138 contains all current complete bacterial, archaeal and viral genomes from RefSeq plus viral
139 neighbor sequences and the human reference genome, and 'nt', which contains all microbial
140 sequences (including fungi and protists) in the non-redundant nucleotide collection nr/nt
141 provided by NCBI (see Suppl. Methods Section 2 for details). The 'std' database furthermore
142 includes the UniVec and EmVec sequence sets of synthetic constructs and vector sequences; and
143 low-complexity k-mers in microbial sequences were masked using NCBI's dustmasker with
144 default settings. We use two metrics to compare how well methods can separate true positives
145 and false positives: (a) F1 score, i. e. the harmonic mean of precision p and recall r, and (b) recall
146 at a maximum false discovery rate (FDR) of 5%. For each method, we compute and select the
147 ideal thresholds based on the read count, k-mer count or abundance calls. Precision p is defined
148 as the number of correctly called species (or genera) divided by the number of all called species
149 (or genera) at a given threshold. Recall r is the proportion of species (or genera) that are in the
150 test dataset and that are called at a given threshold. Higher F1 scores indicate a better separation
151 between true positives and false positives. Higher recall means that more true species can be
152 recovered while controlling the false positives.

153

154 Because the NCBI taxonomy has been updated since the datasets were published, we manually
155 updated the "truth" sets in several datasets (see Suppl. Methods Section 2.3 for all changes). Any
156 cases that might have been missed would result in a lower apparent performance of KrakenHLL.
157 Note that we exclude the over ten-year-old simulated datasets simHC, simMC and simLC from
158 Mavromatis et al. (2007), as well as the biological dataset JGI SRR033547 which has only 100
159 reads.

Data Type	Rank	Statistic	orig			std			nt		
			Reads	Kmers	Diff	Reads	Kmers	Diff	Reads	Kmers	Diff
Bio	Genus	Recall	0.90	0.93	**+4.0%**	0.89	0.94	**+6.2%**	0.91	0.99	**+8.9%**
		F1	0.95	0.96	**+1.8%**	0.95	0.97	**+2.6%**	0.96	0.99	**+3.4%**
	Species	Recall	0.85	0.87	**+2.6%**	0.70	0.78	**+11.8%**	0.95	0.98	**+3.1%**
		F1	0.94	0.94	**+0.7%**	0.90	0.92	**+2.5%**	0.97	0.99	**+1.6%**
Sim	Genus	Recall	0.96	0.94	-2.1%	0.95	0.97	**+2.5%**	0.98	0.99	**+0.8%**
		F1	0.98	0.98	-0.0%	0.98	0.98	**+0.3%**	0.99	0.99	**+0.3%**
	Species	Recall	0.92	0.93	**+0.6%**	0.88	0.88	**+0.3%**	0.90	0.90	-0.1%
		F1	0.97	0.97	**+0.3%**	0.94	0.94	**+0.5%**	0.96	0.96	-0.1%

160

161 Table 1: Performance of read count and unique k-mer thresholds on 10 biological and 21
162 simulated datasets against three databases (‘orig’, ‘std’, ‘nt). Unique k-mer count thresholds give
163 up to 10% better recall and F1 scores, particularly for the biological datasets.

164

165 ***Unique k-mer versus read count thresholds***

166 We first looked at the performance of the unique k-mer count thresholds versus read count
167 thresholds (as would be used with Kraken). The k-mer count thresholds worked very well,
168 particularly for the biological datasets (Table 1 and Suppl. Table 3). On the genus level, the
169 average recall in the biological datasets increases by 4-9%, and the average F1 score increases 2-
170 3%. On the species level, the average increase in recall in the biological sets is between 3 and
171 12%, and the F1 score increases by 1-2%.

172

173 On the simulated datasets, the differences are less pronounced and vary between databases, even
174 though on average the unique k-mer count is again better. However, only in two cases (genus
175 recall on databases 'orig' and 'std') the difference is higher than 1% in any direction. We find
176 that simulated datasets often lack false positives with a decent number of reads but a lower
177 number of unique k-mer counts, which we see in real data. Instead, in most simulated datasets
178 the number of unique k-mers is linearly increasing with the number of unique reads in both true
179 and false positives (Suppl. Figure 4). In biological datasets, sequence contamination and lower
180 read counts for the true positives make the task of separating true and false positives harder.

181

182 ***Comparison of KrakenHLL with eleven other methods***

183 Next, we compared KrakenHLL's unique k-mer counts with the results of eleven metagenomics
184 classifiers from McIntyre et al. [15], which include the alignment-based methods Blast + Megan
185 [16, 17], Diamond + Megan [17, 18] and MetaFlow [19], the k-mer based CLARK [20],
186 CLARK-S [21], Kraken [9], LMAT [22], NBC [23] and the marker-based methods GOTTCHA
187 [24], MetaPhlAn2 [25], PhyloSift [26]. KrakenHLL with database 'nt' has the highest average
188 recall and F1 score across the biological datasets, as shown in Table 2. As seen before, using
189 unique k-mer instead of read counts as thresholds increases the scores. While the database
190 selection proves to be very important (KrakenHLL with database 'std' is performing 10% worse
191 than KrakenHLL with database 'nt'), only Blast has higher average scores than KrakenHLL with
192 k-mer count thresholds on the original database. On the simulated datasets, KrakenHLL with the
193 'nt' database still ranks at the top, though, as seen previously there is more variation (Suppl.

194 Table 4). Notably CLARK is as good as KrakenHLL, but Blast has much worse scores on the

195 simulated datasets.

196

	Genus		Species		
	F1	Recall	F1	Recall	Avg
KrakenHLL nt kmers	**0.99**	**0.99**	**0.99**	**0.98**	**0.99**
KrakenHLL nt reads	0.96	0.91	0.97	0.95	0.95
BlastMeganFilteredLiberal	0.97	0.94	0.97	0.89	0.94
BlastMeganFiltered	0.97	0.93	0.96	0.87	0.93
KrakenHLL orig kmers	0.96	0.93	0.94	0.87	0.93
ClarkM4Spaced	0.95	0.90	0.94	0.88	0.92
KrakenHLL orig reads	0.95	0.90	0.94	0.85	0.91
Kraken	0.95	0.90	0.94	0.84	0.91
KrakenHLL std kmers	0.97	0.94	0.92	0.78	0.90
DiamondMegan_sensitive	0.98	0.93	0.92	0.74	0.89
KrakenFiltered	0.95	0.91	0.90	0.75	0.88
ClarkM1Default	0.94	0.85	0.91	0.77	0.87
KrakenHLL std reads	0.95	0.89	0.90	0.70	0.86
LMAT	0.97	0.93	0.91	0.60	0.85
DiamondMegan	0.94	0.87	0.91	0.66	0.85
Gottcha	0.91	0.84	0.87	0.67	0.82
NBC	0.87	0.76	0.85	0.73	0.80
Metaphlan	0.94	0.89	0.83	0.55	0.80
MetaFlow	0.66	0.53	0.65	0.51	0.59
PhyloSift	0.68	0.29	0.78	0.54	0.57
PhyloSift90pct	0.68	0.30	0.77	0.52	0.57

197

198 Table 2: Performance of KrakenHLL (with unique k-mer count thresholds) compared to

199 metagenomic classifiers [15] on the biological datasets (n=10). F1 and Recall show the average

200 values over the datasets. Note that 'KrakenHLL reads' would be equivalent to standard Kraken.

201

202 **Generating a better test dataset, and selecting an appropriate k-mer threshold**

203 In the previous section we demonstrated that KrakenHLL gives better recall and F1-scores than
204 other classifiers on the test datasets, given the correct thresholds. How can the correct thresholds
205 be determined on real data with varying sequencing depths and complex communities? The test
206 datasets are not ideal for that: The biological datasets lack complexity with a maximum of 25
207 species in some of the samples, while the simulated samples lack the features of biological
208 datasets.

209

210 We thus generated a third type of test dataset by sampling reads from real bacterial isolate
211 sequencing runs, of which there are tens of thousands in the Sequence Read Archive (SRA). That
212 way we created a complex test dataset for which we know the ground truth, with all the features
213 of real sequencing experiments, including lab contaminants and sequencing errors. We selected
214 280 SRA datasets from 280 different bacterial species that are linked to complete RefSeq
215 genomes (see Suppl. Methods Section 2.4). We randomly sampled between one hundred and one
216 million reads (logarithmically distributed) from each experiment, which gave 34 million read
217 pairs in total. Furthermore, we sub-sampled five read sets with between one to twenty million
218 reads. All read sets were classified with KrakenHLL using the 'std' database.

219

220 [FIGURE 3]

221 Figure 3: Unique k-mer count separates true and false positives better than read counts in a
222 complex dataset with ten million reads sampled from SRA experiments. Each dot represents a
223 species, with true species in orange and false species in black. The dashed and dotted lines show
224 the k-mer thresholds for the ideal F1 score and recall at a maximum of 5% FDR, respectively. In

225 this dataset, a unique k-mer count in the range 10000–20000 would give the best threshold for
226 selecting true species.
227
228 Consistent with the results of the previous section, we found that unique k-mer counts provide
229 better thresholds than read counts both in terms of F1 score and recall in all test datasets (e.g.
230 Figure 3 on ten million reads – species recall using k-mers is 0.85, recall using reads 0.76). With
231 higher sequencing depth, the recall increased slightly - from 0.80 to 0.85 on the species level,
232 and from 0.87 to 0.89 on the genus level. The ideal values of the unique k-mer count thresholds,
233 however, vary widely with different sequencing depths. We found that the ideal thresholds
234 increase by about 2000 unique k-mers per one million reads (see Figure 4). McIntyre et al. [15]
235 found that k-mer based methods show a positive relationship between sequencing depths and
236 misclassified reads. Our analysis also shows that with deeper sequencing depths higher
237 thresholds are required to control the false-positive rate.
238
239 [FIGURE 4]

No. of reads	Fraction	Genus Threshold	Genus Recall	Species Threshold	Species Recall
1 million	0.03	2555	0.87	3682	0.80
2 million	0.06	4483	0.86	6152	0.81
5 million	0.15	12723	0.87	10459	0.85
10 million	0.3	21896	0.88	21201	0.85
20 million	0.6	43417	0.88	43417	0.84
34.3 million	1	69847	0.89	688842	0.85

240
241 Figure 4: Deeper sequencing depths require higher unique k-mer count thresholds to control
242 false-positive rate and achieve the best recall. A minimum threshold of about 2000 unique k-mer
243 per a million reads gives the best results in this dataset (solid line in plot).

244

245 In general, we find that for correctly identified species, we obtain up to approximately *L-k*
246 unique k-mers per each read, where *L* is the read length, because each read samples a different
247 location in the genome. (Note that once the genome is completely covered, no more unique k-
248 mers can be detected.) Thus the k-mer threshold should always be several times higher than the
249 read count threshold. For the discovery of pathogens in human patients, discussed in the next
250 section, a read count threshold of 10 and unique k-mer count threshold of 1000 eliminated many
251 background identifications while preserving all true positives, which were discovered from as
252 few as 15 reads.

253

254 **Results on biological samples for infectious disease diagnosis**

255 Metagenomics is increasingly used to find species of low abundance. A special case is the
256 emerging use of metagenomics for the diagnosis of infectious diseases [27, 28]. In this
257 application, infected human tissues are sequenced directly to find the likely disease organism.
258 Usually, the vast majority of the reads match (typically 95-99%) the host, and sometimes fewer
259 than 100 reads out of many millions of reads are matched to the target species. Common skin
260 bacteria from the patient or lab personnel and other contamination from sample collection or
261 preparation can easily generate a similar number of reads, and thus mask the signal from the
262 pathogen.

263

264 To assess if the unique k-mer count metric in KrakenHLL could be used to rank and identify
265 pathogen from human samples, we reanalyzed ten patient samples from a previously described
266 series of neurological infections [4]. That study sequenced spinal cord mass and brain biopsies

267 from ten hospitalized patients for whom routine tests for pathogens were inconclusive. In four of
268 the ten cases, a likely diagnosis could be made with the help of metagenomics. To confirm the
269 metagenomics classifications, the authors in the original study re-aligned all pathogen reads to
270 individual genomes.
271
272 Table 3 shows the results of our reanalysis of the confirmed pathogens in the four patients,
273 including the number of reads and unique k-mers from the pathogen, as well as the number of
274 bases covered by re-alignment to the genomes. Even though the read numbers are very low in
275 two cases, the number of unique k-mers suggests that each read matches a different location in
276 the genome. For example, in PT8, 15 reads contain 1570 unique k-mers, and re-alignment shows
277 2201 covered base pairs. In contrast, Table 4 shows examples of identifications from the same
278 datasets that are not well-supported by k-mer counts. We also examined the likely source of the
279 false positive identifications by blasting the reads against the full nt database, and found rRNA of
280 environmental bacteria, human RNA and PhiX-174 mis-assignments (see Suppl. Methods for
281 details). Notably, the common laboratory and skin contaminants PhiX-174, *Escherichia coli*,
282 *Cutibacterium acnes* and *Delftia* were detected in most of the samples, too (see Suppl. Table 6).
283 However, those identifications are solid in terms of their k-mer counts - the bacteria and PhiX-
284 174 are present in the sample, and the reads cover their genomes rather randomly. To discount
285 them, comparisons against a negative control or between multiple samples is required (e.g. with
286 Pavian [29]).
287
288 Table 3: Validated pathogen identifications in patients with neurological infections have high
289 numbers of unique k-mers per read. The pathogens were identified with as few as 15 reads, but

290 the high number of unique k-mers indicates distinct locations of the reads along their genomes.
291 Re-alignment of mapped reads to their reference genomes (column "Covered Bases")
292 corroborates the finding of the unique k-mers (see also Suppl. Figure 5). Interestingly, the k-mer
293 count in PT5 indicates that there might be multiple strains present in the sample since the k-mers
294 cover more than one genome. Read lengths were 150-250 bp.
295
296

Sample	Matched microorganism	Reads	K-mers	Covered Bases
PT5	Human polyomavirus 2	9,650	7,129	5,130 / 5,130
PT7	*Elizabethkingia genomosp. 3*	403	20,724	53,256 / 4,433,522
PT8	*Mycobacterium tuberculosis*	15	1,570	2,227 / 4,411,532
PT10	Human gammaherpesvirus 4	20	2,084	2,822 / 172,764

297
298 Table 4: False positive identifications have few unique k-mers. Using an extended taxonomy, the
299 identifications in PT4 and PT10 were matched to single accessions (instead of to the species
300 level). The likely true source of the mapped sequences was determined by subsequent BLAST
301 searches and included 16S rRNA present in many uncultured bacteria, human small nucleolar
302 RNAs (snRNAs), and phiX174.

Sample	Matched microorganism	Reads	K-mers	Source
PT3	*Clostridioides difficile*	122	126	16S rRNA
PT4	Hepatitis C virus JF343788.1 Recombinant Hepatitis C virus	101	3	Human snRNA
PT5	*Akkermansia muciniphila*	936	136	16S rRNA
PT10	Human betaherpesvirus 5 JN379815.1 Human herpesvirus 5 strain U04, partial genome	63	5	phiX174

303
304

305 **Further extensions in KrakenHLL**

306 KrakenHLL adds three further notable features to the classification engine.

307 1. Enabling strain identification by extending the taxonomy: The finest level of granularity
308 for Kraken classifications are nodes in the NCBI taxonomy. This means that many strains
309 cannot be resolved, because up to hundreds of strains share the same taxonomy ID.
310 KrakenHLL allows extending the taxonomy with virtual nodes for genomes,
311 chromosomes and plasmids, and thus enabling identifications at the most specific levels
312 (see Suppl. Methods Section 3)
313 2. Integrating 100,000 viral strain sequences: RefSeq includes only one reference genome
314 for most viral species, which means that a lot of the variation of viral strain is not covered
315 in a standard RefSeq database. KrakenHLL sources viral strain sequences from the NCBI
316 Viral Genome Resource that are validated as 'neighbors' of RefSeq viruses, which leads
317 to up to 20% more read classifications (see Suppl. Methods Section 4).
318 3. Hierarchical classification with multiple databases. Researcher's may want to include
319 additional sequence sets, such as draft genomes, in some searches. KrakenHLL allows to
320 chain databases and match each k-mer hierarchically, stopping when it found a match.
321 For example, to mitigate the problem of host contamination in draft genomes, a search
322 may use the host genome as first database, then complete microbial genomes, then draft
323 microbial genomes. More details are available in Suppl. Method Section 5.

324

325 **Timing and memory requirements**

326 The additional features of KrakenHLL come without a runtime penalty and very limited
327 additional memory requirements. In fact, due to code improvements, KrakenHLL often runs

328 faster than Kraken, particularly when most of the reads come from one species. On the test
329 dataset, the mean classification speed in million base-pairs per minute increased slightly from
330 410 to 421 Mbp/m (see Suppl. Table 3). When factoring in the time needed to summarize
331 classification results by kraken-report, which is required for Kraken but part of the classification
332 binary of KrakenHLL, KrakenHLL is on average 50% faster. The memory requirements increase
333 on average by 0.5 GB from 39.5 GB to 40 GB.
334
335 On the pathogen Id patient data, where in most cases over 99% of the reads were either assigned
336 to human or synthetic reads, KrakenHLL was significantly faster than Kraken (Suppl. Table 5).
337 The classification speed increased from 467 to 733 Mbp/m. The average wall time was about
338 44% lower, and the average additional memory requirements were less than 1GB, going from
339 118.0 to 118.4 GB. All timing comparisons were made after preloading the database and running
340 with 10 parallel threads.
341

342 Discussion

343 In our comparison, KrakenHLL performed better in classifying metagenomics data than many
344 existing methods, including the alignment-based methods Blast [16], Diamond [30], and
345 MetaFlow [19]. Blast and Diamond results were post-processed by Megan [31], which assigns
346 reads to the lowest-common ancestor (LCA), but ignores coverages when computing the
347 resulting taxonomic profile. Thus, the taxonomic profile (with read counts as abundance
348 measures) is sensitive to over-representing false positives that have coverage spikes in parts of
349 the genome in the same way as non-alignment based methods. Coverage spikes may appear due
350 to wrongly matched common sequences (e.g. 16S rRNA), short amplified sequences floating in

351 the laboratory, and contamination in database sequences. MetaFlow, on the other hand,
352 implements coverage-sensitive mapping, which should give better abundance calls, but it did not
353 perform very well in our tests. Going from alignments to a good taxonomic profile is difficult
354 because coverage information cannot be as easily computed for the LCA taxon and summarized
355 for higher levels in the taxonomic tree. In comparison, reads and unique k-mer counts can be
356 assigned to the LCA taxa, and summed to higher levels. Notably, KrakenHLL's k-mer counting
357 is affected by GC biases in the sequencing data the same way as other read classifiers and
358 aligners [32], and may underreport GC-rich or GC-poor genomes.
359

360 # Conclusions

361 KrakenHLL is a novel method that combines fast k-mer based classification with an efficient
362 algorithm for counting the number of unique k-mers found in each species in a metagenomics
363 dataset. When the reads from a species yield many unique k-mers, one can be more confident
364 that the taxon is truly present, while a low number of unique k-mers suggests a possible false
365 positive identification. We demonstrated that using unique k-mer counts provides improved
366 accuracy for species identification, and that k-mer counts can help greatly in identifying false
367 positives. In our comparisons with multiple other metagenomics classifiers on multiple
368 metagenomics datasets, we found that KrakenHLL consistently ranked at the top. The strategy of
369 counting unique k-mer matches allows KrakenHLL to detect that reads are spread across a
370 genome, without the need to align the reads. By using a probabilistic counting algorithm,
371 KrakenHLL is able to match the exceptionally fast classification time of the original Kraken
372 program with only a very small increase in memory. The result is that KrakenHLL gains many of
373 the advantages of alignment at a far lower computational cost.

Declarations

Availability of data and material

KrakenHLL 🐙 is implemented in C++ and Perl. Its source code is available at https://github.com/fbreitwieser/krakenhll, licensed under GPL3. The version used in the manuscript is permanently available under https://doi.org/10.5281/zenodo.1252385. Analysis scripts for the results of this manuscript are available at https://github.com/fbreitwieser/krakenhll-manuscript-code.

The datasets of McIntyre et al. are available at https://ftp-private.ncbi.nlm.nih.gov/nist-immsa/IMMSA. The sequencing datasets of Salzberg et al. are available under the BioProject accession PRJNA314149 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA314149). Note that human reads have been filtered. The test datasets generated by sampling reads from bacterial isolate SRA experiments are available at ftp://ftp.ccb.jhu.edu/pub/software/krakenhll/SraSampledDatasets.

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Authors' contributions

FPB conceived and implemented the method. FPB and SLS wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

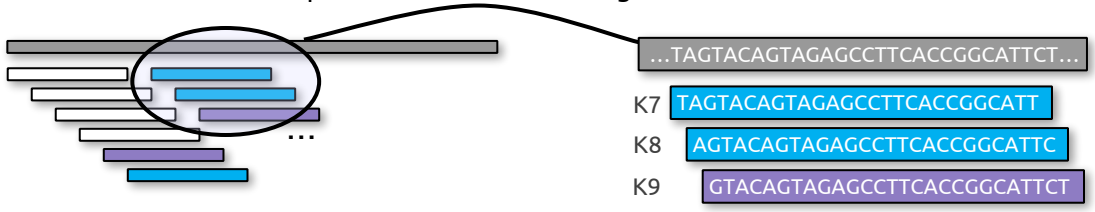
1. Breitwieser FP, Lu J, Salzberg SL: **A review of methods and databases for metagenomic classification and assembly.** *Brief Bioinform* 2017.
2. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, Turner P, Parkhill J, Loman NJ, Walker AW: **Reagent and laboratory contamination can critically impact sequence-based microbiome analyses.** *BMC Biol* 2014, **12:**87.
3. Thoendel M, Jeraldo P, Greenwood-Quaintance KE, Yao J, Chia N, Hanssen AD, Abdel MP, Patel R: **Impact of Contaminating DNA in Whole-Genome Amplification Kits Used for Metagenomic Shotgun Sequencing for Infection Diagnosis.** *J Clin Microbiol* 2017, **55:**1789-1801.

4. Salzberg SL, Breitwieser FP, Kumar A, Hao H, Burger P, Rodriguez FJ, Lim M, Quinones-Hinojosa A, Gallia GL, Tornheim JA, et al: **Next-generation sequencing in neuropathologic diagnosis of infections of the nervous system.** *Neurol Neuroimmunol Neuroinflamm* 2016, **3:**e251.
5. Brown JR, Bharucha T, Breuer J: **Encephalitis diagnosis using metagenomics: application of next generation sequencing for undiagnosed cases.** *Journal of Infection* 2018.
6. Mukherjee S, Huntemann M, Ivanova N, Kyrpides NC, Pati A: **Large-scale contamination of microbial isolate genomes by Illumina PhiX control.** *Stand Genomic Sci* 2015, **10:**18.
7. Dadi TH, Renard BY, Wieler LH, Semmler T, Reinert K: **SLIMM: species level identification of microorganisms from metagenomes.** *PeerJ* 2017, **5:**e3138.
8. Quince C, Walker AW, Simpson JT, Loman NJ, Segata N: **Shotgun metagenomics, from sampling to analysis.** *Nat Biotechnol* 2017, **35:**833-844.
9. Wood DE, Salzberg SL: **Kraken: ultrafast metagenomic sequence classification using exact alignments.** *Genome Biol* 2014, **15:**R46.
10. Flajolet P, Fusy É, Gandouet O, Meunier F: **HyperLogLog: the analysis of a near-optimal cardinality estimation algorithm.** In *AofA: Analysis of Algorithms*; *2007-06-17; Juan les Pins, France*. Discrete Mathematics and Theoretical Computer Science; 2007: 137-156.
11. Heule S, Nunkesser M, Hall A: **HyperLogLog in practice.** 2013**:**683.
12. Ertl O: **New Cardinality Estimation Methods for HyperLogLog Sketches.** *arXiv:170607290* 2017.
13. Brister JR, Ako-adjei D, Bao Y, Blinkova O: **NCBI Viral Genomes Resource.** *Nucleic Acids Research* 2015, **43:**D571-D577.
14. Irber Junior LC, Brown CT: **Efficient cardinality estimation for k-mers in large DNA sequencing data sets.** *bioRxiv* 2016.
15. McIntyre ABR, Ounit R, Afshinnekoo E, Prill RJ, Hénaff E, Alexander N, Minot SS, Danko D, Foox J, Ahsanuddin S, et al: **Comprehensive benchmarking and ensemble approaches for metagenomic classifiers.** *Genome Biology* 2017, **18**.
16. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: **Basic local alignment search tool.** *J Mol Biol* 1990, **215:**403-410.
17. Huson DH, Auch AF, Qi J, Schuster SC: **MEGAN analysis of metagenomic data.** *Genome Res* 2007, **17:**377-386.
18. Buchfink B, Xie C, Huson DH: **Fast and sensitive protein alignment using DIAMOND.** *Nat Methods* 2015, **12:**59-60.
19. Sobih A, Tomescu AI, Mäkinen V: **MetaFlow: Metagenomic Profiling Based on Whole-Genome Coverage Analysis with Min-Cost Flows.** In *Research in Computational Molecular Biology.* 2016: 111-121: *Lecture Notes in Computer Science*].
20. Ounit R, Wanamaker S, Close TJ, Lonardi S: **CLARK: fast and accurate classification of metagenomic and genomic sequences using discriminative k-mers.** *BMC Genomics* 2015, **16:**236.
21. Ounit R, Lonardi S: **Higher classification sensitivity of short metagenomic reads with CLARK-S.** *Bioinformatics* 2016, **32:**3823-3825.

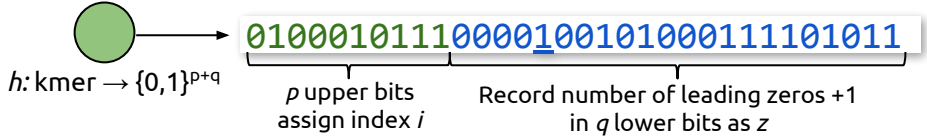
466 22. Ames SK, Hysom DA, Gardner SN, Lloyd GS, Gokhale MB, Allen JE: **Scalable**
467 **metagenomic taxonomy classification using a reference genome database.**
468 *Bioinformatics* 2013, **29:**2253-2260.
469 23. Rosen GL, Reichenberger ER, Rosenfeld AM: **NBC: the Naive Bayes Classification**
470 **tool webserver for taxonomic classification of metagenomic reads.** *Bioinformatics*
471 2010, **27:**127-129.
472 24. Freitas Tracey Allen K, Li P-E, Scholz MB, Chain Patrick SG: **Accurate read-based**
473 **metagenome characterization using a hierarchical suite of unique signatures.**
474 *Nucleic Acids Research* 2015, **43:**e69-e69.
475 25. Truong DT, Franzosa EA, Tickle TL, Scholz M, Weingart G, Pasolli E, Tett A,
476 Huttenhower C, Segata N: **MetaPhlAn2 for enhanced metagenomic taxonomic**
477 **profiling.** *Nat Methods* 2015, **12:**902-903.
478 26. Darling AE, Jospin G, Lowe E, Matsen FAt, Bik HM, Eisen JA: **PhyloSift: phylogenetic**
479 **analysis of genomes and metagenomes.** *PeerJ* 2014, **2:**e243.
480 27. Simner PJ, Miller S, Carroll KC: **Understanding the Promises and Hurdles of**
481 **Metagenomic Next-Generation Sequencing as a Diagnostic Tool for Infectious**
482 **Diseases.** *Clinical Infectious Diseases* 2017.
483 28. Zhang C, Cleveland K, Schnoll-Sussman F, McClure B, Bigg M, Thakkar P, Schultz N,
484 Shah MA, Betel D: **Identification of low abundance microbiome in clinical samples**
485 **using whole genome sequencing.** *Genome Biology* 2015, **16**.
486 29. Breitwieser FP, Salzberg SL: **Pavian: Interactive analysis of metagenomics data for**
487 **microbiomics and pathogen identification.** *BioRxiv* 2016.
488 30. Buchfink B, Xie C, Huson DH: **Fast and sensitive protein alignment using**
489 **DIAMOND.** *Nature Methods* 2014, **12:**59-60.
490 31. Huson DH, Auch AF, Qi J, Schuster SC: **MEGAN analysis of metagenomic data.**
491 *Genome Research* 2007, **17:**377-386.
492 32. Xu Y, Chen Y-C, Liu T, Yu C-H, Chiang T-Y, Hwang C-C: **Effects of GC Bias in Next-**
493 **Generation-Sequencing Data on De Novo Genome Assembly.** *PLoS ONE* 2013, **8**.
494

495

A Read k-mers are looked-up in the database and assigned to taxa:



B For each taxon a data sketch records its k-mers for cardinality estimation



The maximum number of leading zeros are recorded in registers M

Estimated number of unique values for register $M[i]$: ~ $2^{M[i]}$

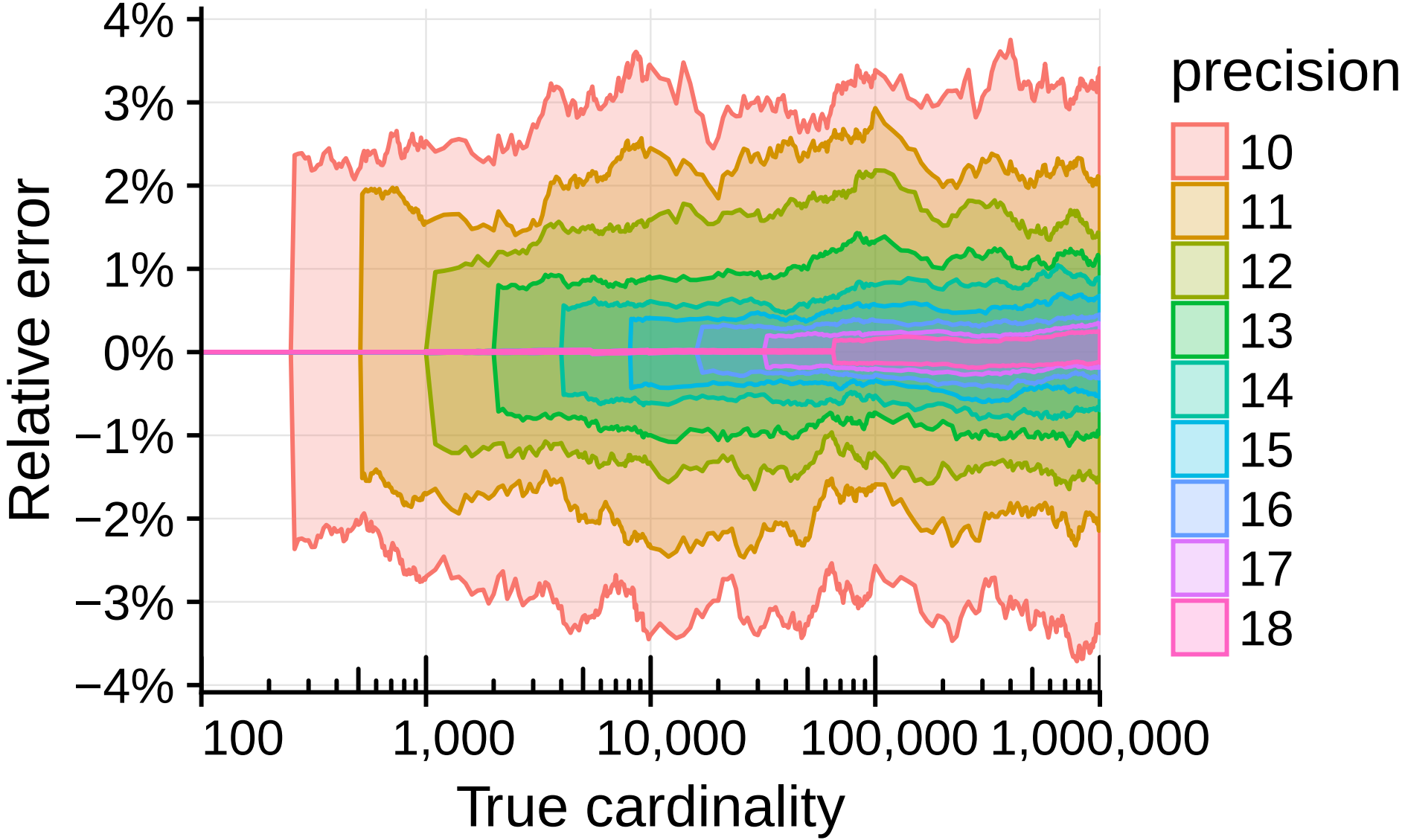
C K-mer count and coverage in taxonomic report show evidence behind classifications:

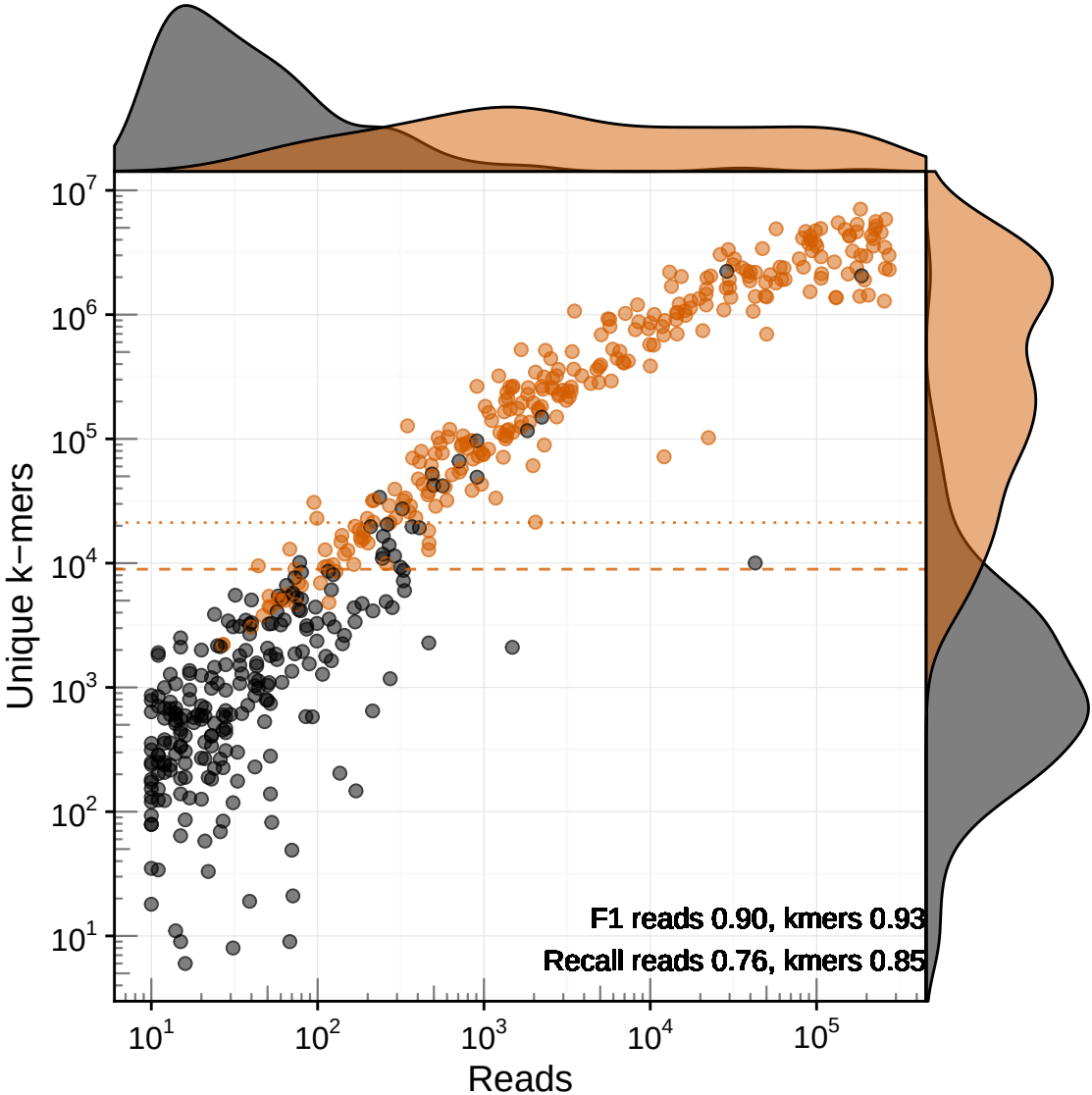
reads	kmers	dup	cov	taxID	rank	name
122	112	144	0.0004	11855	species	*Clostridioides difficile*
9650	7129	74.5	0.192	10632	species	Human polyomavirus 2
15	1570	1	0.0002	7643	species group	*Mycobacterium tb* complex

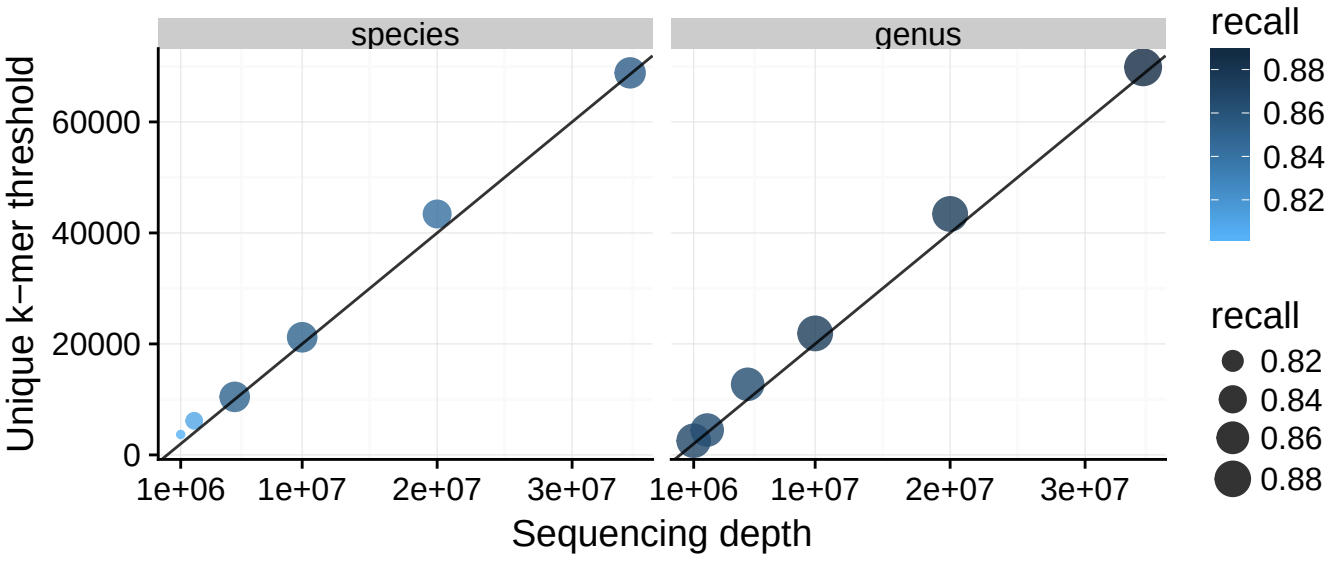
Bad classification with few k-mers

Good classification, reads cover genome

Number of distinct k-mers for taxon, and coverage of the taxon's k-mers


4%
3%
2%
1%
0%
−1%
−2%
−3%
−4%
Relative error
100
1,000
10,000
100,000
1,000,000
True cardinality
precision
10
11
12
13
14
15
16
17
18


F1 reads 0.90, kmers 0.93
Recall reads 0.76, kmers 0.85
Unique k−mers
Reads
10^1
10^2
10^3
10^4
10^5
10^6
10^7


species
genus
Unique k−mer threshold
0
20000
40000
60000
1e+06
1e+07
2e+07
3e+07
Sequencing depth
recall
0.88
0.86
0.84
0.82
recall
0.82
0.84
0.86
0.88