Title: Memory-bound k-mer selection for large and evolutionary diverse reference libraries

Running title: Efficient k-mer selection for large references

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ABSTRACT

Using k-mers to find sequence matches is increasingly used in many bioinformatic applications, including metagenomic sequence classification. The accuracy of these downstream applications relies on the density of the reference databases, which, luckily, are rapidly growing. While the increased density provides hope for dramatic improvements in accuracy, scalability is a concern. The k-mers are kept in the memory during the query time, and saving all k-mers of these ever-expanding databases is fast becoming impractical. Several strategies for subsampling k-mers have been proposed, including minimizers and finding taxon-specific k-mers. However, we contend that these strategies are inadequate, especially when reference sets are taxonomically imbalanced, as are most microbial libraries. In this paper, we explore approaches for selecting a fixed-size subset of k-mers present in an ultra-large dataset to include in a library such that the classification of reads suffers the least. Our experiments demonstrate the limitations of existing approaches, especially for novel and poorly sampled groups. We propose a library construction algorithm called KRANK (K-mer RANKer) that combines several components, including a hierarchical selection strategy with adaptive size restrictions and an equitable coverage strategy. We implement KRANK in highly optimized code and combine it with the locality-sensitive-hashing classifier CONSULT-II to build a taxonomic classification and profiling method. On several benchmarks, KRANK k-mer selection dramatically reduces memory consumption with minimal loss in classification accuracy. We show in extensive analyses based on CAMI benchmarks that KRANK outperforms k-mer-based alternatives in terms of taxonomic profiling and comes close to the best marker-based methods in terms of accuracy.

INTRODUCTION

The number of genomes available in public repositories has been growing dramatically in recent years, especially due to increased sequencing of microbial and viral species. Of the 322,193 bacterial species deposited on RefSeq since 2000, $>$15% have been added in 2023 alone. A major benefit of having access to all these genomes is to build ultra-large reference libraries with the potential to search new *query* sequences against them. Such libraries can be used to classify reads from a metagenomic sample and to detect contaminants. It has been long appreciated that the accuracy of these downstream applications in metagenomics relies on having access to dense reference libraries as classification accuracy suffers when the query is distant from all reference genomes (Meijenfeldt et al., 2019; Pachiadaki et al., 2019; Rachtman, Balaban, et al., 2020; Liang et al., 2020). Many authors have recently attempted to create ultra-large genomic reference sets (e.g., Parks et al., 2018; McDonald et al., 2023), often used with marker genes (Asnicar et al., 2020; Balaban et al., 2023). Using these datasets with k-mers-based methods, however, runs into a mundane but key limitation — the memory needed to utilize these genomes as reference libraries.

Using k-mers to build ultra-large libraries and classify reads has been a promising approach, as evident from the success of Kraken-II (Wood et al., 2019). Kraken-II and other methods such as CLARK (Ounit and Lonardi, 2016) and CONSULT-II (Şapcı et al., 2024) extract k-mers for some large k (e.g., 31) from the reference set, use some strategy to choose a subset of k-mers, and build a searchable data structure (e.g., a hash table) of the selected k-mers and their taxonomic associations. At the time of the query, the entire data structure is loaded into the memory, k-mers from reads are extracted and searched against the data structure, and a read is classified at some taxonomic rank if certain heuristic conditions (specific to each method) are met. It is easy to store millions or even a few billion k-mers in memory but modern datasets can include tens or hundreds of billions of k-mers. For example, Şapcı et al. (2024) were able to fit 8 billion 32-mers and their taxonomic labels into $\approx$140Gb of memory,

but these had to be subsampled (arbitrarily) from 20 billion 32-mers available in the moderate-size database of 10,575 genomes produced several years ago by Zhu et al. (2019). Trade-offs of accuracy and scalability imposed by the need to subsample k-mers will be increasingly felt by developers of k-mer matching methods and those aiming to build ultra-large reference sets.

For the k-mer-based methods to benefit from the ever-growing set of available genomes, we need improved methods of subsampling k-mers in a memory-bound fashion. Of course, we are not the first to note the need to subsample k-mers. Minimization is the standard technique that selects some among adjacent k-mers (Zheng et al., 2023; Roberts et al., 2004) and is adopted by most tools. However, we argue that beyond minimization, which focuses on the redundancy of adjacent k-mers, we need to consider the evolutionary dimension. This direction can be stated as a computational problem: We are given a taxonomic tree $\mathcal{T}$ and a set of genomes $\mathcal{G}$ labeled by the taxonomy. Let $\mathcal{K}$ be the set all of distinct k-mers across all genomes $g \in \mathcal{G}$. We seek a subset of $\mathcal{K}$ with size $M \leq |\mathcal{K}|$ such that the reference taxonomy is represented well with the selected k-mers. Users can adjust M to control the memory budget (see METHODS for details).

The problem statement leaves the meaning of "well-represented" unspecified as many criteria can be considered, and the choice of the criterion forms the basis of each method. A notable attempt in this direction is pioneered by Lee et al. (2011) who propose seeking discriminative k-mers specific to a species not found in others, an idea implemented in CLARK (Ounit and Lonardi, 2016). Another obvious approach is to represent all reference genomes at equal levels, or similarly, to sample k-mers uniformly at random. The overall goal of this work is to explore these and a set of alternative strategies in the context of taxonomic read classification and taxonomic profiling of metagenomic samples.

Subsampling k-mers from ultra-large and heterogeneous databases available these days faces many challenges. On the one hand, sampling of taxonomic groups in public libraries is very imbalanced, which can become a challenge as noted by Nasko et al. (2018). For example, RefSeq includes 35947 *Escherichia* genomes while 1164 genera have only a single genome. In the dataset of Zhu et al. (2019) (WoL-v1 hereafter), despite their attempts to find representative species, the number of genomes sampled per taxon in each taxonomic rank varies three orders of magnitude (Fig. 1A). The true imbalance in evolutionary history (where some clades are much more diverse, abundant, and species-rich than others) in addition to biases in sampling (where some environments are sampled far more than others) are behind such imbalances. Regardless of the cause, a naive subsampling of k-mers can easily miss entire branches of the evolutionary tree. The situation is made worse by the fact that for $k \gg 20$, a large number of k-mers are not shared even among genomes of the same species. Examining WoL-v1 dataset, we see that 15% of pairs of genomes from the same species have pairwise distances above 5% (Fig. 1B), which results in 98% of 30-mers being unique to each genome in such pairs (Fig. 1C). Pairs of genomes from the same genus but different species shared more than 10% of their 30-mers in only 1% of cases. Thus, as the number of genomes N grows, the number of unique k-mers grows close to proportionally to N. Moreover, k-mers unique to ranks above species will be far outnumbered by those unique to a genome or a species. As a result, optimal k-mer selection is even more challenging if one tries to find a unique set of k-mers to be used at all ranks (perhaps the reason Ounit and Lonardi (2016) target a particular rank).

In this paper, we propose a set of strategies for selecting a predefined number of k-mers from a large pool of genomes in ways that are conducive to classifying reads. Our goal is to find subsets that do not leave out poorly sampled groups, work across taxonomic ranks, and do not reduce the ability to classify relatively novel reads. We implement and explore these strategies paired with the read classification and taxonomic profiling method CONSULT-II. Our method, k-mer RANKer (KRANK), takes a taxonomy and a set of genomes labeled with the taxonomy as input. It selects a subset of k-mers from these genomes based on its ranking strategy and a user-defined size constraint. KRANK subsets k-mers in a top-down traversal of the reference taxonomy, enabling it to make choices about

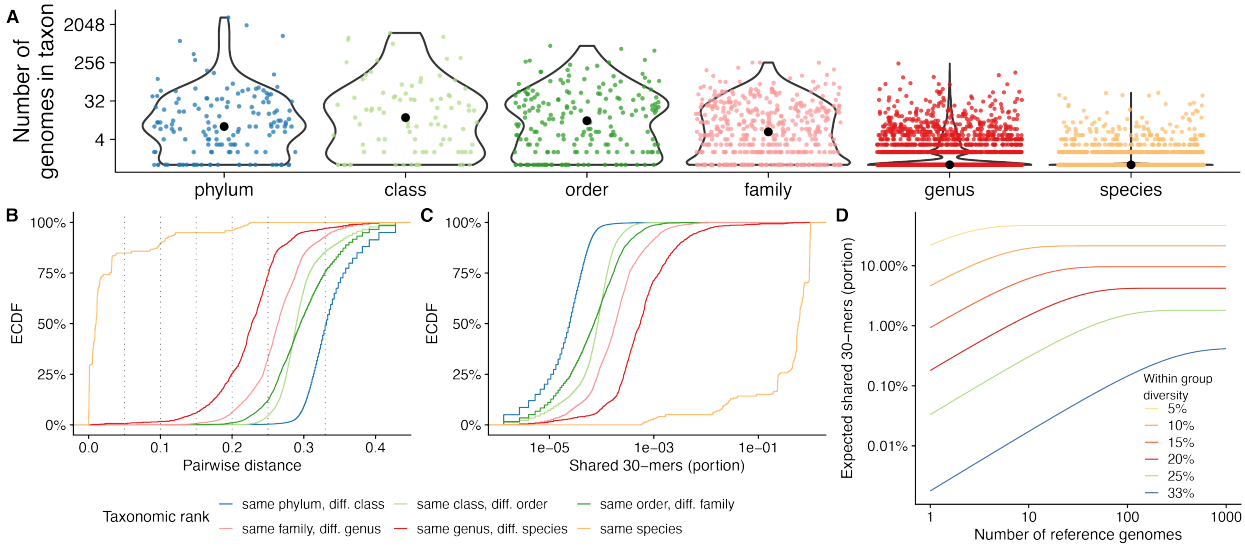


Figure 1: A) Number of genomes in WoL-v1 (Zhu et al., 2019) reference set under each taxonomic node (dots), separated by ranks. B,C) The distribution of Mash (Ondov et al., 2016) estimated genomic distances (B) and Jaccard similarities (C) among 500,000 randomly sampled pairs of genomes that share a taxonomic rank but are different in lower ranks. The empirical cumulative distribution function (ECDF) is shown. D) The theoretical expectation for the number of 30-mers shared between a query and at least one of N sampled genomes of a reference set for a group that has within-group diversity $2d$ is shown as $(1-d)^k\left(1-(1-(1-d)^k)^N\right)$.

what k-mers to keep locally instead of globally and eliminating the need to analyze all k-mers jointly at any point in library construction. Our highly optimized and flexible C++ implementation allowed us to compare several alternative strategies empirically. Our comprehensive results on taxonomic read classification and taxonomic profiling tasks show that KRANK, unlike more naive selection strategies, can produce dramatic reductions in library sizes without substantial loss of accuracy.

RESULTS

We designed KRANK (detailed in Algorithm 1) to select a predefined number of k-mers from a given set of genomes. KRANK designates some taxonomic rank as the leaf rank (species by default). It then traverses the taxonomy $\mathcal{T}$ in post-order. At leaves, distinct k-mers are extracted and encoded in a compact manner (see Algorithmic details of KRANK). Following CONSULT-II, KRANK indexes k-mers using l locality-sensitive hashing (LSH) tables. These LSH tables ensure two k-mers with a small Hamming distance (e.g., less than 3) have a high chance of being indexed to the same row in at least one of the tables (see LSH tables for details). This is achieved by selecting $h < k$ random but fixed positions of a k-mer as its hash key, and each k-mer is stored in the row of the table indexed by the LSH key. As we traverse the tree, on each internal node, we adaptively impose a size constraint on its tables. We combine each row of the hash tables of all the children nodes and remove k-mers based on a ranking algorithm until the size constraint is satisfied. At the root, the final l tables are restricted to b columns per row, and each will contain $2^{2h}b$ k-mers. When not specified, we use $l = 2$, $k = 32$, $h = 12$, $b = 16$. Given the hash tables at the root, we use the existing CONSULT-II algorithm for taxonomic classification and abundance profiling (see CONSULT-II classification and profiling).

The heart of the algorithm is adaptive size constraints and k-mer ranking, both of which we em-

pirically motivate first. These explanatory sets of results use a manageable microbial dataset by Zhu et al. (2019) composed of 10,575 genomes (WoL-v1 hereafter). We chose 756 query genomes in total (676 bacterial and 80 archaeal genomes), of which only 10 are present in the reference set. Measuring the novelty by the minimum distance of a query to any reference genome as estimated by Mash (d^*), we bin queries into novelty groups (Fig. S1A). We use 150bp error-prone reads simulated from each genome (66667 reads per query genome) and test methods in terms of their accuracy in classifying individual reads. See Datasets and Evaluation metrics for details. After exploratory results, we move to more formal evaluations of the method for two tasks: taxonomic classification of metagenomic reads and taxonomic profiling of metagenomic samples.

KRANK: motivating the design

Adaptive size constraints

We observed wide differences among taxa, even at the same rank, in terms of sampling density (Fig. 1A) and levels of diversity (Fig. 1B). This heterogeneity creates a major challenge in selecting k-mers. If we randomly sample k-mers, taxa will contribute proportionally to their sampling in the final subset. Given a total budget M, in expectation, a taxon t would have $M|\mathcal{K}_t|/|\mathcal{K}|$ k-mers in the sampled subset, where $\mathcal{K}_t$ is the set of k-mers of all genomes labeled by taxon t, and $\mathcal{K}$ is the set of all reference k-mers. As a result, taxa with lower sampling will have little representation, while highly-sampled groups (e.g., *E. coli*) will dominate. We partially address this challenge by imposing a size constraint on $|\mathbf{H}_t|$ for each internal node during traversal to avoid having bloated nodes that starve sister taxa. While this balancing can in theory be done at the root, we would need to keep a map from k-mers to genomes, which is impractical. Thus, preemptively filtering some number of k-mers from some tables helps scalability by reducing the memory usage. We designed a heuristic for adaptive size constraints whereby, each node t containing portion $r(t)$ of the total dataset gets a budget of $\sqrt{r(t)}M$ k-mers. We define $r(t)$ as either the portion of total k-mers present under t (default) or the proportion of taxonomic leaves (i.e., species) under t (see Section AdaptiveSizeConstraint for details). We empirically evaluate these two versions of this heuristic against the baseline of enforcing no adaptive constraint, all paired with random k-mer subsampling.

Imposing the adaptive size constraints can make a substantial difference for taxonomic groups with low levels of sampling (Fig. 2A). For example for phyla with $[0, 50]$ and $(50, 500]$ reference genomes sampled, both definitions of $r(t)$ improve the F1 accuracy over enforcing no adaptive constraint for up to 35% and 12%, respectively, with similar improvements obtained at other ranks. Thus, the adaptive size constraints achieve their stated goal of ensuring groups with lower sampling are not left out. This equitable spreading of the k-mer budget inevitably results in reduced representation for highly sampled groups. For example, for the few phyla with more than 500 reference genomes, we observe a considerable drop in the performance (e.g., F1 score declines by 14%–20%). Thus, it may appear that better sampling of low-representation groups has to come at the expense of the high-representation groups. However, this tradeoff can be avoided.

Since we have two different tables ($l = 2$) in the library, we can have a mixed strategy: We enforce the adaptive size constraint for one table but not the other. This mixed strategy is almost as good as the adaptive version for low-representation groups, and almost as good as the no-adaptive-constraint strategy for highly sampled groups (Fig. 2A). Overall, this mixed strategy outperforms having no adaptive constraints, both in terms of precision and recall (Fig. 2B). The improvements are most visible for moderately novel queries ($d^* \in (0.025, 0.1]$). Across queries, the mixed strategy improves the recall by 6%–10% and precision by 2%–5% above the genus rank. Thus, KRANK adopts the mixed strategy as the default.

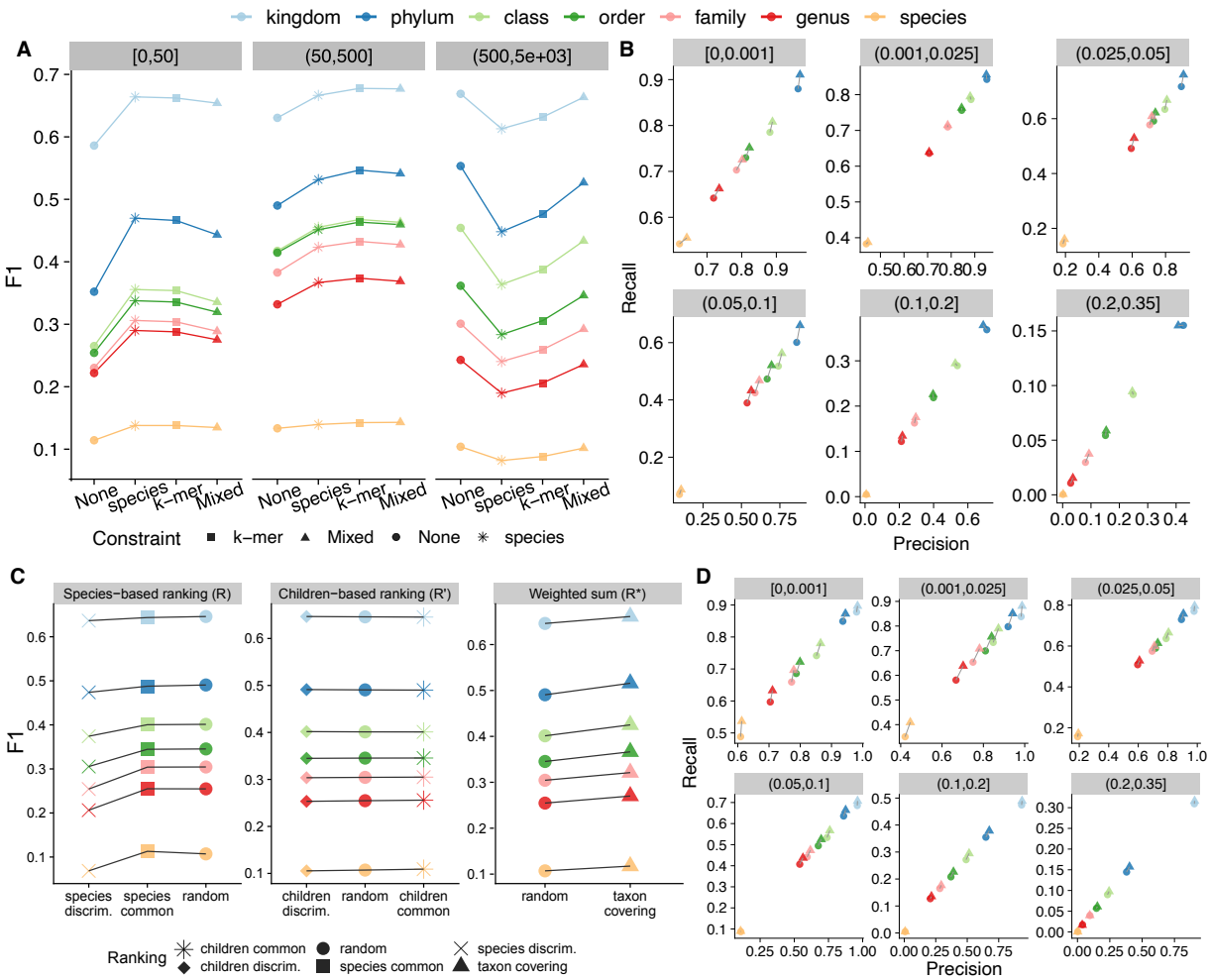


Figure 2: A, B) Comparison of adaptive size-constraint approaches with random k-mer selection. None: enforcing no constraint, species: number of species constraint, k-mer: total k-mer count constraint, Mixed: no constraint for one table and k-mer count constraint for the other. A) F1 accuracy, dividing queries (panels) into three bins based on the number of reference genomes in the phylum of the query. B) Precision vs recall of None vs Mixed (default), dividing queries based bins of query novelty (d^*). C,D) Evaluation of simple ranking strategies (discriminative, common, random) implemented using species counts R or children counts R' and our new weighted-sum R^* heuristic (11). See Fig. S2 for an illustration of functions. C) F1 across all queries. D) Precision versus recall of random and weighted-sum heuristic, dividing queries by bins of novelty (d^*). In A) and C) x-axes are ordered by the average F1 score of all queries. All results use: $w = 35$, $k = 32$, $l = 2$, $h = 12$, and $b = 16$. d^* is the distance to the closest reference estimated by Mash. Default KRANK uses mixed adaptive size and R^* ranking.

Ranking and removing k-mers

Faced with the question of which k-mers to select given the limited budget, three options immediately present themselves: selecting randomly, selecting *discriminative* k-mers unique to some taxa, or conversely, selecting common k-mers present in many taxa. We compared these options and others that we propose paired with the adaptive size constraint approach described above using constraints for both tables (i.e., not mixed). In implementing these strategies, we used two quantities — the number of leaves (i.e., species) under taxon t that include a k-mer x denoted by $\mathrm{R}(x, t)$, and the number of

children of t that include the k-mer x at least once denoted by $\mathrm{R}'(x,t)$, as shown in Fig. S2.

The case against discriminative k-mers. Keeping discriminative k-mers found only in specific target species, an approach followed by Ounit, Wanamaker, et al. (2015), would correspond to preferentially filtering out k-mers that appear in more than one species (or some other rank), which corresponds to $\mathrm{R}(x,t) > 1$. Going one step further, we emulate the discriminative k-mer strategy by ranking k-mers inversely to $\mathrm{R}(x,t)$ or $\mathrm{R}'(x,t)$, thus removing common k-mers. Empirically, using either version is worse than simply using random selection (Fig. 2C). The reduction is negligible with children-based $\mathrm{R}'(x,t)$ but is quite dramatic when used with species counts $\mathrm{R}(x,t)$; for example, the declines in F1 are 36% and 19% for species and genus ranks, respectively. Thus, we caution against using discriminative k-mers in a memory-bound setting.

The reduced accuracy of selecting unique k-mers can be explained by noting that very few k-mers are shared even among different species of the same genus. For example, in the WoL-v1 data set (Zhu et al., 2019), a random sample of genomes that are from different species but the same genus share only 0.05% of their 30-mers (Fig. 1C) and have Mash distances that typically exceed 20% (Fig. 1B). With simplifying assumptions, we can approximate the probability that a k-mer would be shared between a query genome and at least one of N genomes sampled from a taxonomic group, assuming each pair of genomes in this group has distance d to parent, as $(1-d)^k\left(1-(1-(1-d)^k)^N\right)$. Plotting this equation shows that using discriminative k-mers leads to very few matches between a query and the reference (Fig. 1D). For instance, in a group with $2d = 20\%$ diversity (which is less than most genera; see Fig. 1B), only 0.7% of query 30-mers are expected to be found in at least one among $N = 5$ references genomes and only 4.2% when $N \to \infty$ (an infinite number of genomes sampled from that genus). Since most k-mers are unique and subsampling is inevitable in a memory-bound setting, removing common k-mers makes it difficult to find k-mers matches between reads and reference species that have 20% or more distance to them (e.g., when the closest available match is at the genus level). This only gets worse at higher ranks and is not much better within the same species, where the pairwise Mash distance is 5% or more in 15% of cases.

Selecting common k-mers alone does not help. We can emulate common k-mer selection by ranking k-mers proportionally to $\mathrm{R}(x,t)$ or $\mathrm{R}'(x,t)$. Given the argument against discriminative k-mers, common k-mers seem appealing because they can represent each taxon using conserved sequences present across different genomes. Empirically, this strategy used with either $\mathrm{R}(x,t)$ or $\mathrm{R}'(x,t)$ is better than using discriminative k-mers but is no better than random sampling (Fig. 2C). Just like randomly selecting k-mers, this strategy focuses the budget on larger and densely sampled taxa. For instance, the WoL-v1 reference set includes 2975 *Pseudomonadota* genomes and only a single genome from many other phyla. While some unevenness is undoubtedly due to genuine differences among the diversity of phyla, some must be due to uneven sampling.

Covering all children improves accuracy. Instead of maximizing total coverage, we can attempt to cover all species. We designed a scalable heuristic ranking mechanism to fulfill this goal. We rank k-mers by a weighted sum of $R(x,.)$ values of t's children (see method section FilterByRank and $R^*(x,t)$ defined in Eq. (11) and illustrated in Fig. S2). The weights are used to de-emphasize children that are highly sampled among surviving k-mers. We simply set the weights to be inversely proportional to the *coverage* of each child taxon among k-mers of a particular row of the LSH table to ensure that children covered with fewer surviving k-mers get covered by the parent. Using this approach improves k-mer selection consistently across all taxonomic ranks compared to the random strategy and common k-mer strategy (Fig. 2C). Further dividing queries by their novelty, we observe that our weighted sum strategy improves both precision and recall, particularly for less novel queries; recall increases by as much as 15% at the species level in the $d^* \in (0.001, 0.25]$ bin (Fig. 2D).

Comparison to other methods for taxonomic read classification

The default version of KRANK uses both adaptive size constraints (mixed strategy) and k-mer ranking based on Eq. (11) and comes in two default modes: high-sensitivity (hs) using 51.2Gb and lightweight (lw) using 12.8Gb. We next compare these two versions of KRANK against CONSULT-II, CLARK, and Kraken-II on the same WoL-v1 dataset used so far in terms of accuracy in classifying individual reads (Fig. 3).

Accuracy in default settings. Across different ranks and query novelty levels, KRANK-hs matched or improved on CONSULT-II in terms of F1 accuracy despite using far fewer k-mers and about $1/3$ of the memory (Fig. 3A). KRANK-hs and CONSULT-II often had similar precision (with a slight advantage for CONSULT-II in some cases), but KRANK-hs had better recall in many settings (Fig. S3). KRANK-lw is more accurate than CONSULT-II at the kingdom level and only slightly less accurate elsewhere despite using less than ten times the memory. Accuracy also depended on query novelty (d^*). All methods had similar F1 accuracy for queries that were not novel ($d^* \leq 0.025$), except CLARK which had slightly lower accuracy. For these less novel queries, both variants of KRANK were indistinguishable from CONSULT-II. For more novel queries ($d^* > 0.025$), accuracy dropped for all methods and substantial differences between them emerged. For example, for the $d^* \in (0.05, 0.1]$ bin, KRANK-lw outperformed Kraken-II and CLARK above the species rank, achieving 16% and 35% higher F1 scores, respectively, at the phylum level. Unlike KRANK-hs, KRANK-lw had slightly lower accuracy than CONSULT-II at some ranks (e.g., 8%–9% at the order rank) for $d^* \in (0.025, 0.1]$ and moderately lower accuracy (e.g., 12% at the phylum rank) for $d^* > 0.1$. Nevertheless, KRANK-lw outperforms Kraken-II (e.g., 77% and 250% higher F1 at the phylum rank for $d^* \in (0.1, 0.2]$ and $(0.2, 0.35]$, respectively).

Both KRANK-hs and KRANK-lw are slightly better than CONSULT-II in terms of kingdom-level classification for novel genomes. This improvement can be due to the better representation of archaea with careful k-mer selection. Note that our query set is highly imbalanced (e.g., 90% bacteria and 33% *Pseudomonadota*). Thus, simply assigning all reads to the largest group (in terms of the number of query genomes) at each rank would still perform well (Fig. S4). As expected, this null model performs well at the kingdom level (0.94 F1 score). However, KRANK (both lw and hs versions) is the only method that improves on the null model (0.96 and 0.97 average F1 scores, respectively) and assigns 83% of archaeal reads to archaea. At lower ranks, all tools, except for CLARK at the phylum rank, do substantially better than the null model.

Adjusting memory usage. We next explored three to seven levels of consumed memory for KRANK, CONSULT-II, and Kraken-II to compare their accuracy given similar levels of memory, ranging from 1.6Gb to 140Gb (Fig. 3B). KRANK clearly and dramatically outperformed both alternatives in terms of average F1 score when memory is controlled. At lower memory levels, CONSULT-II had much lower F1 scores than KRANK, and Kraken-II had lower F1 than CONSULT-II for more novel queries. For instance, given $\leq$4Gb, KRANK was 26% more accurate than CONSULT-II and 8% more accurate than Kraken-II for the least novel bin. For the moderately novel queries ($d^* \in (0.05, 0.1]$), KRANK with 3.2Gb outperformed Kraken-II with 46.5Gb. KRANK with 1.6Gb outperformed CLARK (149.6Gb) and Kraken-II (46.5Gb) for novel queries ($d^* > 0.1$), giving 160% and 89% higher F1 scores, respectively. Note that CONSULT-II and KRANK store the same number of k-mers (268M in a single table) when memory usages are 5Gb and 3.2Gb, respectively. KRANK remained more accurate than CONSULT-II when we compared the method for varying numbers k-mers kept in the library (Figs. S5 and S6). Since the only difference between KRANK and CONSULT-II is the selected k-mers, these results demonstrate the effectiveness of the selection algorithm.

Resource usages. Compared to CONSULT-II, KRANK built libraries using considerably less computational resources due to its efficient implementation and despite performing more complex computations

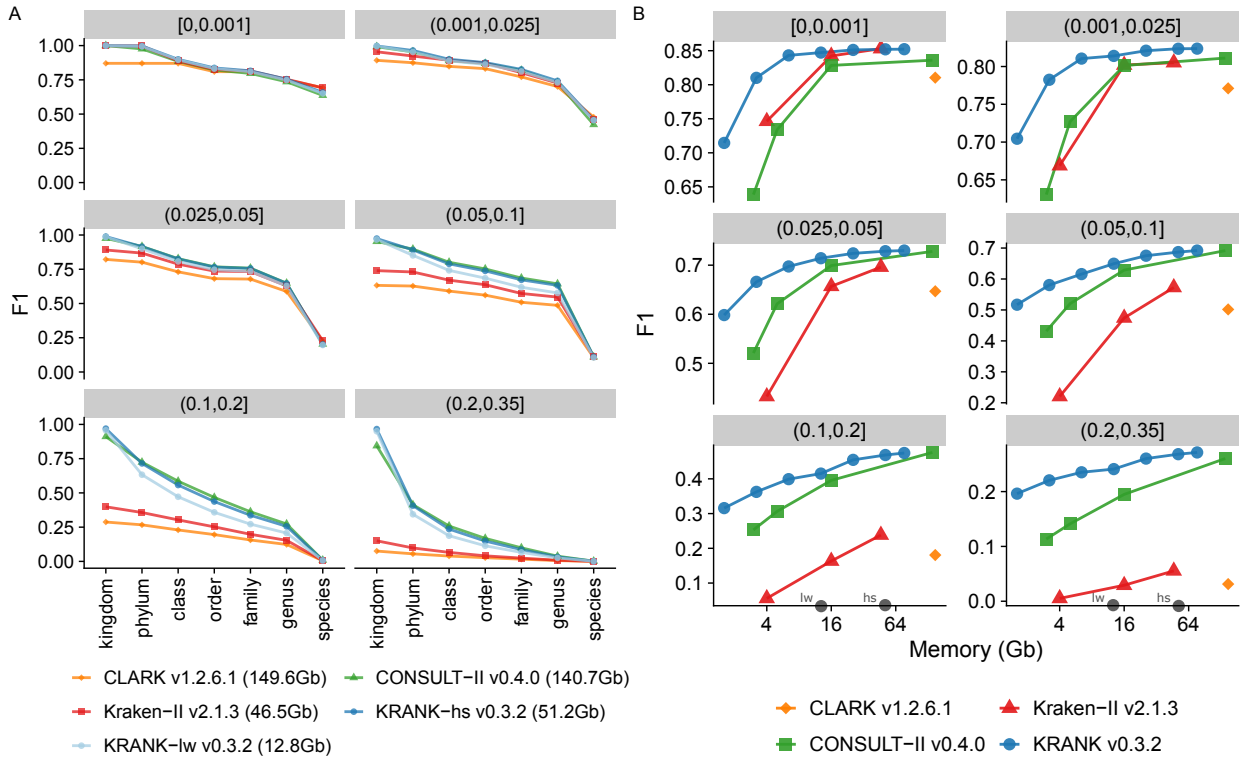


Figure 3: A) Comparison of two default modes of KRANK (lw for lightweight and hs for high-sensitivity) with other methods across ranks and novelty bins on the WoL-v1 dataset with 66667 simulated 150bp reads from each of 756 query genomes. Each panel is a novelty bin, corresponding to the minimum distance to any reference genome (d^*), measured by Mash (Ondov et al., 2016); see Fig. S1. F1 scores are averages of all queries in each d^* bin. See Fig. S3 for precision and recall. B) F1 accuracy for KRANK, CONSULT-II, and Kraken-II as we change the memory used in Gb. CLARK is shown as a single data point. On the x-axis, we mark conditions corresponding to the lw and hs modes.

(Table 1). While the total time needed was slightly less, the main gain is KRANK's distributed memory implementation. KRANK splits the LSH table into batches, enabling it to divide the workload into independent jobs that can be run on different nodes. For instance, on this dataset, KRANK-hs was built using 512 batches per table, each of which took 12 minutes on average with 14 threads. KRANK-lw took half the time (6 minutes per batch). Using this configuration, the peak memory usage for each batch was less than 32 GB. Processing 36 batches in parallel (e.g., on different cluster nodes) would require 3.5 hours to complete the library construction. While building their libraries, Kraken-II and CLARK used $\approx$4.5 times less CPU hours than KRANK-hs.

At the query time, compared to CONSULT-II, KRANK does not introduce any extra computation but its smaller libraries result in reduced running times. On this dataset, KRANK-hs was more than twice as fast as CONSULT-II (Table 1). These improvements are due to better cache performance and shorter library loading times. Note, however, that Kraken-II was around 15$\times$ faster than KRANK-hs. This difference may be due to the fact that KRANK needs to compute HD for up to $2b = 14$ reference k-mers for each query k-mer whereas Kraken-II only computes presence/absence.

	Running time		Peak Memory	
	Query	**Library**	**Query**	**Library**
Kraken-II	88 sec, 96 threads	3.5 hr, 96 threads	47Gb	50Gb
CLARK	676 sec, 96 threads	10 hr, 32 threads	150Gb	390Gb
CONSULT-II	2914 sec, 96 threads	19 hr, 128 threads	141Gb	157Gb
KRANK-hs	1291 sec, 96 threads	3 hr, 36×14 threads	51Gb	32Gb per batch
KRANK-lw	1187 sec, 96 threads	1.5 hr, 36×14 threads	13Gb	8Gb per batch

Table 1: Computational resources needed for a database built on 10,470 reference genomes and 756 queries (52M short reads). Measurements for queries were performed using 96 threads on a machine with 2.2 GHz AMD EPYC 7742 processors. Library building time for KRANK used 36 batches, each 14 threads, distributed across multiple cluster nodes and run in parallel. KRANK and CONSULT-II library construction does not include k-mer extraction, which took 45 minutes with 14 threads for KRANK.

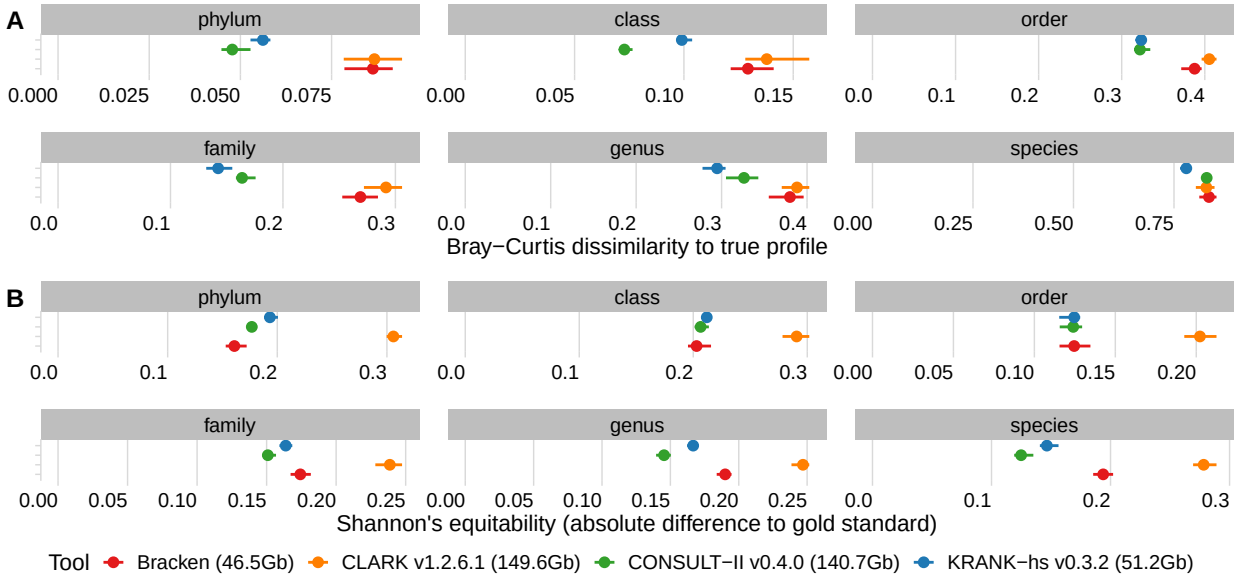


Figure 4: Taxonomic profiling on CAM-I challenge. A) The Bray-Curtis metric measures the dissimilarity between the estimated and true profiles. B) The difference between Shannon's equitability of the estimated profile and of the gold standard, measuring how well each tool reflects the alpha diversity (i.e., the evenness of taxon abundances). We show the mean and minimum maximum across five samples.

Abundance profiling: CAMI challenges

CAMI-I high complexity dataset. We next compared KRANK-hs against three alternatives in terms of taxonomic profiling accuracy on the Critical Assessment of Metagenome Interpretation (CAMI-I) (Sczyrba et al., 2017) benchmarking challenge, focusing on the high complexity subset. We compared against CONSULT-II, CLARK, and Bracken (a Bayesian extension of Kraken-II) using standard metrics promoted by CAMI-I (see Datasets and Evaluation metrics). All methods were run with the custom libraries built using the same WoL-v1 dataset used in the previous analysis.

Abundance profiles estimated by KRANK-hs and CONSULT-II are the most similar to the true profile

in terms of Bray-Curtis dissimilarity (Fig. 4A). At the species level, all methods have high errors and are comparable, with a slight advantage for KRANK-hs. As we move up the ranks, the error quickly drops for all methods, but KRANK-hs and CONSULT-II outperform others. Overall, KRANK-hs performs similarly to CONSULT-II despite the much-reduced memory. KRANK performs considerably better than both Bracken and CLARK. All methods tend to underestimate Shannon's equitability, which is a measure of the variety and distribution of taxa present in a sample. At the phylum rank, Bracken has closer values to the gold standard, while CONSULT-II and KRANK become better at the family rank or lower (Fig. 4B). Overall, KRANK-hs is slightly less accurate than CONSULT-II in terms of this metric across ranks. Note that correcting for genome size improves the accuracy of both CONSULT-II and KRANK, but interestingly KRANK benefits slightly more in terms of the Bray-Curtis metric (Fig. S7A). Since the largest three phyla in our reference set constitute 80%–85% of each sample, this dataset presents a case where the KRANK strategy of covering low-sampled groups is not needed and sampling of highly covered phyla by CONSULT-II is sufficient.

CAMI-II marine and strain-madness datasets. To compare KRANK to a larger set of methods, we built a library based on 72,766 genomes available on RefSeq as of 2019/01/08. Since this same dataset had been provided to all methods tested in the CAMI-II challenge (Meyer, Fritz, et al., 2022), we were able to compare KRANK with 12 alternative methods from the original paper, in addition to CONSULT-II, which was also applied to this dataset (see Datasets). These methods include both marker-based and k-mer-based methods.

Taxonomic profiles of KRANK-hs were among the most accurate on the CAMI-II challenge (Fig. 5). On the strain-madness dataset, in which only 2 phyla constitute 90% of the relative abundance, our method was a close second to the best method, which was the marker-based method MetaPhlAn (Figs. 5 and S8A). Averaged across all samples, the UniFrac score of KRANK-hs was only 2% less than MetaPhlAn and 6% above the third-best method, CONSULT-II. In species, class, and phylum ranks, MetaPhlAn had slightly better L1 than KRANK-hs, while in the family rank, KRANK-hs was slightly better (Fig. 5). Despite using the same profiling algorithm as CONSULT-II and $1/3$ of its memory, KRANK-hs also had better L1 metrics than CONSULT-II across all ranks on this dataset. Other k-mer-based methods such as Bracken had far lower accuracy in terms of both metrics.

On the marine dataset, which includes both archaeal and bacterial genomes, and consists of 65%–70% *Proteobacteria*, the pattern slightly changed (Fig. 5). Here, CONSULT-II was slightly more accurate than KRANK-hs, with 3% better UniFrac and 3% better L1, averaged over all ranks and samples. Only at the species level KRANK-hs was more accurate than CONSULT-II (6% in terms of L1). Overall, KRANK-hs was ranked fourth, following mOTU, CONSULT-II, and MetaPhlAn (Fig. S8). Note, however, that the best method, mOTU, performed poorly on the strain-madness dataset. KRANK-hs was the second best k-mer-based method after CONSULT-II but used only $1/3$ of its memory. The third leading k-mer-based method, Bracken, was less accurate than CONSULT-II and KRANK-hs in both metrics, but with a smaller margin than the strain-madness dataset.

To summarize, KRANK-hs was the best k-mer-based method and the second best method overall (Fig. S8B). Averaged over both datasets, the marker-based method MetaPhlAn had the best accuracy, followed closely by KRANK-hs, and then CONSULT-II. Note, however, that we focused on the two main CAMI-II metrics that accounted for abundance values. Judged by the presence/absence of taxa (i.e., ignoring abundance), KRANK-hs had better purity than CONSULT-II and Bracken and about the same level of completeness (Fig. S9). However, these abundance-agnostic metrics were not the strength of k-mer-based methods; across both datasets, KRANK-hs, CONSULT-II, and Bracken were among the best in terms of completeness but not purity.

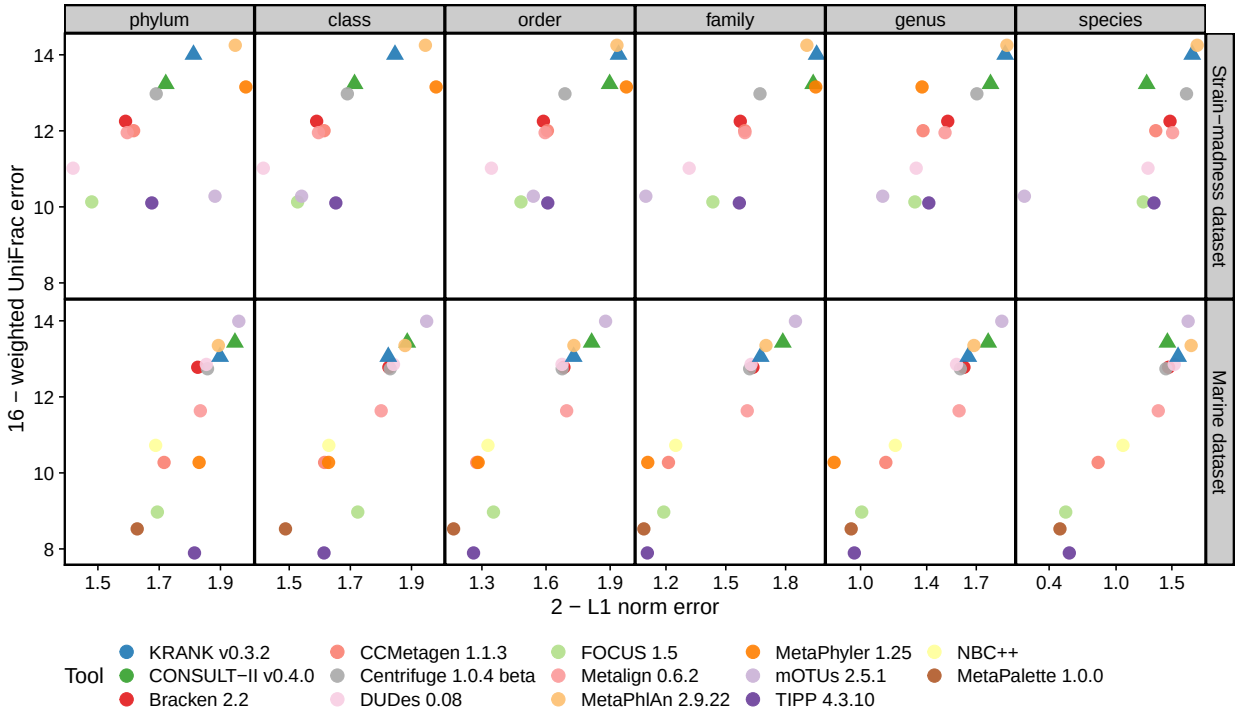


Figure 5: Comparing KRANK with other participants in CAMI-II benchmarking challenge (Meyer, Fritz, et al., 2022). As in the original manuscript, we show the upper bound of L1 norm (2) minus actual L1 norm versus the upper bound of weighted UniFrac error (16) minus actual weighted UniFrac error. Each data point stands for the average of 100 strain-madness samples or 10 marine samples. Metrics were computed using OPAL with default settings and the `-n` option. The UniFrac error is the total amount of predicted abundances that needs to be moved along the edges of the taxonomic tree to make them overlap with the true abundance profile. The L1 error simply measures the accuracy of reconstructing the relative abundance profile at a fixed rank.

DISCUSSION

We introduced KRANK for selecting a memory-bound and representative subset of k-mers from a large genome collection for the purpose of taxonomic classification and abundance profiling. We used a tree-based k-mer filtering algorithm that decides which k-mers to remove as it moves up a taxonomic tree. This bottom-up strategy makes the library construction more memory efficient as k-mers are analyzed in smaller chunks at each node. In addition, we use an adaptive size constraint mechanism to leave some filtering decisions to higher taxonomic levels. These decisions are made based on the prevalence of k-mers among children of or leaves under each node.

Ultimately, both our adaptive size constraints and our ranking strategies are heuristics, with no theoretical guarantees, but designed based on careful experiments involving novel queries and unbalanced sampling. Due to the empirical nature of the method, the exploration of alternatives will be an ongoing topic of research, one that is helped by KRANK software design. In particular, our approach still leaves room for ties during ranking, and smarter ways to break such ties may help accuracy. Alternatively, more theoretically justified approaches should be explored.

One obstacle to building a more theoretical approach is to define an objective function for k-mer selection. Subtleties of unbalanced and biased sampling in reference databases make the definition of the desired optimization problem tricky. Should we attempt to combat the sampling bias by selecting

k-mers more proportionally from under-sampled groups? If so, how can we know and model the bias? Should we attempt to consider likely profiles of queries? If so, under what model? These questions have no easy answers. Nevertheless, defining mathematically rigorous but nuanced objectives for k-mer selection could be a fruitful avenue of future research.

Our approach focused on k-mer similarity along the evolutionary dimension but completely ignored k-mer overlap. Selecting k-mers based on overlap is well covered by minimizers, which we use as a preprocessing step. However, our ranking algorithm is unaware of overlap and minimizers are unaware of the taxonomic relationships. Integrating the evolutionary-aware strategies of KRANK with location-based minimizers poses an interesting algorithmic challenge and could further improve the method. However, designing such a combination is not trivial given our hashing strategy and needs further algorithmic developments.

Despite all the improvements compared to CONSULT-II, the library construction of KRANK is expensive in terms of CPU hours spent. Nevertheless, the creation of the reference library is a one-time operation, amortized over many subsequent uses. Furthermore, computation is getting cheaper rapidly, and the 1500 CPU hours we used for the WoL-v1 (or 3400 CPU hours for the CAMI-II database) are far from prohibitive. Crucially, the batching strategy enables us to build the library over a cluster (i.e., distributed memory), with each job using as little as 8Gb and many jobs running in parallel (Table 1). Our analyses of 72,766 genomes included in the CAMI-II case demonstrate the scalability of the method to scales commensurate with modern-day libraries.

As datasets become even larger, KRANK parameters may need to adjust. Many ultra-large reference databases are available now, including GTDB (Parks et al., 2018) with 402,709 genomes, and WoL-v2 (Balaban et al., 2023), with a phylogeny built from 199,330 genomes. Moreover, RefSeq currently includes millions of genomes. The fast increase is primarily due to the sequencing of many more genomes from key species. As the number of genomes from each species increases, our choice in this paper to treat the species rank as the leaves of the taxonomy becomes less scalable. However, KRANK can easily use a more refined group (e.g., subspecies) as the leaf, which will trade off slightly longer running times for better memory efficiency. It could also enable sub-species-level classification, a topic that should be explored in the future.

METHODS

As a k-mer selection method, KRANK can, in principle, be paired with any k-mer-based classification or profiling methods. However, in this paper, we use KRANK solely with CONSULT-II (Şapcı et al., 2024); k-mer matches found in libraries built by KRANK are given as input to CONSULT-II `v0.5.0` for classification and profiling. Moreover, our k-mer selection algorithm adopts a similar LSH data structure to CONSULT-II. Thus, we start by describing the CONSULT-II hash tables and the CONSULT-II classification and profiling methods before presenting the details of KRANK.

LSH tables

CONSULT(-II) was designed to answer the following question: Are there any k-mers in a given reference set with Hamming distances (HD) less than some threshold p to a given query k-mer? The core idea is to find low HD matches efficiently by partitioning reference k-mers to constant-sized subsets (i.e., rows of the hash table with size b) using the bit-sampling LSH method (Har-Peled et al., 2012). In this method, we select h random but fixed positions of a k-mer as its hash key. Each LSH table is a simple $2^{2h} \times b$ array, and l such hash tables constitute the data structure (default: $l = 2$, h=14, and b=16 for KRANK).

Each query is compared against all $l \times b$ k-mers with the same hash key. We return the closest k-mer match with distance $\leq p$ if any exists. Since we explicitly compute the HD of the query to these

reference k-mers, there are no false positive matches but false negatives are possible. Using the bit sampling scheme (Har-Peled et al., 2012), one can guarantee that two k-mers at HD$=d$ have the same hash with probability $(1 - d/k)^h$ assuming independence of positions. Thus, given two k-mers, the probability that *at least one* of l LSH keys is the same for both k-mers is given by

$$\rho(d) = 1 - \left(1 - \left(1 - \frac{d}{k}\right)^h\right)^l . \tag{1}$$

For small enough p (e.g., p=4), the probability $\rho(d)$ drops quickly when $d \gg p$ and it is sufficiently high if $d \leq p$. Furthermore, since classification is done at the read level, there will be $L - k + 1$ chances for a k-mer match for a read of length L. For such a read, the expected number of matching k-mers is $(L - k + 1)\rho(d)$, which can be large enough for several realistic choices of l and h (Fig. S10). For instance, with k=30, h=14 and l=2 (KRANK-hs settings), for a 150bp read at 15% distance from the closest reference genome, we would still expect 23.5 k-mer matches *if all of those reference k-mers are in the database*. If only 20% of the k-mers from the reference genome are in the library, we still expect more than 5 k-mer matches, which can be sufficient for classification. In practice, the number of matches will depend on the budget M, and will necessarily decrease as we reduce M.

CONSULT-II extends LSH tables further by adding a taxonomic label for each k-mer in the table. This label is the lowest common ancestor (LCA) of a *subset* of species that have the corresponding k-mer (hence the name *soft*-LCA). This subset is determined with a probabilistic procedure that performs a Bernoulli trial for each genome and includes its species in the subset if the trial is successful. The success probability is different for each k-mer x_i and is a function of the number of genomes in which the k-mer appears (N_i): $p_u(N_i) = w \log_2(2^{\frac{N_i - 1}{s}} + 2)^{-1}$, where w and s are hyperparameters.

CONSULT-II classification and profiling

Both read classification and profiling algorithms of CONSULT-II are based on voting. Given a query read $\mathcal{R}$, CONSULT-II lets each k-mer $x \in \mathcal{R}$ vote for the soft-LCA of the reference k-mer it matches. The value of the vote decreases as the HD between the query k-mer x and its best match increases. Let the best match be at distance d to a k-mer with soft-LCA at taxon t; then, x votes $\mathrm{v}_x(t) = \left(1 - \frac{d}{k}\right)^k$ units for t. To take hierarchical relations in the taxonomic tree into account, CONSULT-II aggregates votes by recursively summing them in a bottom-up manner. Let $\mathrm{child}(t)$ be the set of children of the taxon t. Then, the total vote of taxon t for read $\mathcal{R}$ is:

$$\bar{\mathrm{v}}_{\mathcal{R}}(\mathrm{t}) = \sum_{x \in \mathcal{R}} \mathrm{v}_x(t) + \sum_{t' \in \mathrm{child}(t)} \bar{\mathrm{v}}_{\mathcal{R}}(\mathrm{t}') \ . \tag{2}$$

For read classification, CONSULT-II simply determines a majority vote threshold τ, corresponding to half of the total vote value at the root of the tree. It then assigns each read to the group at the lowest rank whose total vote value strictly exceeds τ. Note that this choice is guaranteed to be unique.

To derive an abundance profile, CONSULT-II first normalizes the total vote values of a read $\mathcal{R}_i$ per each taxonomic rank using

$$\mathrm{v}^*_{\mathcal{R}_i}(t) = \frac{\bar{\mathrm{v}}_{\mathcal{R}_i}(t)}{\sum_{t' \in \mathcal{T}_r} \bar{\mathrm{v}}_{\mathcal{R}_i}(t')} \tag{3}$$

where $\mathcal{T}_r$ is the set of all taxa at rank r (e.g., all phyla). Next, it gathers normalized total vote values of all n reads $\mathcal{R}_1, \ldots, \mathcal{R}_n$ in a sample, and normalizes again to obtain the final profile. Let $\mathrm{p}^r = \left[p^r_t\right]_{t \in \mathcal{T}_r}$ denote the relative read abundance profile at rank r, summing up to 1. CONSULT-II (since `v0.3.0`)

sets the relative abundance of taxon t to:

$$p_t^r = \frac{\sum_i^n \mathrm{v}^*_{\mathcal{R}_i}(t)}{\sum_{t' \in \mathcal{T}_r} \sum_i^n \mathrm{v}^*_{\mathcal{R}_i}(t')} \,. \tag{4}$$

Note that p_t^r estimates the ratio of *reads* belonging to the taxon t, but we are often interested in the relative abundances of *cells* (denoted by $\hat{\mathrm{p}}^r = \left[\hat{p}_t^r\right]_{t \in \mathcal{T}_r}$). Thus, CONSULT-II (since `v0.4.0`) incorporates genome sizes by simply correcting p_t^r values as follows:

$$\hat{p}_t^r = \frac{p_t^r l_t^{-1}}{\sum_{t' \in \mathcal{T}_r} p_t^r l_{t'}^{-1}} \tag{5}$$

where l_t is the average genome length of all references in taxon t. See Fig. S7A for a comparison between `v0.3.0` corresponding to Eq. (4) and `v0.4.0` implementing Eq. (5) on the CAMI-I dataset. As an option, CONSULT-II can replace the denominator of Eq. (4) with the total vote at the root of the taxonomic tree to quantify the abundance of unclassified taxa. This can be viewed as propagating vote values down from a parent to an artificial "other" lineage which continues until the species rank.

As opposed to classification, CONSULT-II's profiling (`v0.4.0`) algorithm does not require a majority vote, and hence, a read can contribute to abundance values of multiple taxa at a fixed rank, even when there exist only a few high HD matches. Furthermore, normalized values can accumulate over billions of reads in a sample, and can result in erroneous profiles (potentially explaining relatively high errors of CONSULT-II at species rank in CAMI-II strain-madness dataset 4). In `v0.5.0` (first introduced in the present manuscript), CONSULT-II introduces a new profiling algorithm, which uses a very similar principle to its classification algorithm. Keeping the genome size correction (Eq. (5)) the same, `v0.5.0` modifies Eq. (4) as follows:

$$p_t^r = \frac{1}{n} \sum_i^n \mathbb{1}\{\mathrm{v}^*_{\mathcal{R}_i}(t) > \max(\tau, \tau_0)\} \tag{6}$$

where τ is the total vote value of the taxonomic tree and τ_0 is a hyperparameter hard threshold (default: 0.03). In words, instead of each k-mer of a read voting for a different taxon, each read votes collectively; the read votes for the same taxon where CONSULT-II would classify it if its total vote is high enough (i.e., $> \tau_0$). Reads with total votes below τ_0 and few or ambiguous matches are considered unclassified. Thus, the resulting abundance profile does not have to sum to 1. Using `v0.5.0` instead of `v0.4.0` with KRANK's library resulted in improvements in terms of Bray-Curtis dissimilarity especially at lower ranks (Fig. S7). Thus, we report profiling results using `v0.5.0` for KRANK in both CAMI challenges.

Algorithmic details of KRANK

We use $\mathcal{T}$ to denote the set of all nodes of the taxonomy when clear by context. We let $\mathcal{S} \subseteq \mathcal{T}$ be the set of species, and for $t \in \mathcal{T}$, we let $\mathcal{S}_t \subset \mathcal{S}$ be the set of species under node t. We can choose the leaves of the tree $\mathcal{T}$ to correspond to any desired rank, but by default, we use species ($\mathcal{S}$) as the lowest rank; this can be easily changed to any other rank. Let $\mathcal{K}_t$ denote the set of k-mers of all genomes labeled by a taxon $t \in \mathcal{T}$. Notice that, $\mathcal{K}_t = \cup_{t' \in \mathcal{S}_t} \mathcal{K}_{t'}$.

Recall that during the tree traversal of Algorithm 1, for each node $t \in \mathcal{T}$, three key operations are performed to build a new hash table denoted by $\mathbf{H}_t$:

1. UNIONTABLES: takes the union of hash tables of its children, $\mathbf{H}_{t'}, t' \in \mathrm{child}(t)$,
2. ADAPTIVESIZECONSTRAINT: computes a size constraint for $\mathbf{H}_t$,
3. FILTERBYRANK: ranks and removes k-mers from the union until it fits the size constraint.

At the root, the library is flattened to a 1D array (PROJECTTABLE). We first describe our two main heuristics and then discuss details of other named functions in Algorithm 1.

Algorithm 1 KRANK algorithm. Functions are discussed in the text.

```
procedure BuildLibrary(t)
    H_t ← an empty table with 2^{2h} rows
    if t ∈ S then
        K_t ← ExtractKmers(t)
        H_t ← FillTable(K_t)
        return H_t
    for all t' ∈ child(t) do
        H_{t'} ← BuildLibrary(t')
    for all t' ∈ child(t) do
        H_t ← UnionTables(H_{t'}, H_t)
    M(t) ← AdaptiveSizeConstraint(t)
    n ← |H_t| − M(t)
    if n > 0 then
        FilterByRank(H_t, n)
    if t = root then
        ProjectTable(H_t)
    return H_t
```

AdaptiveSizeConstraint

Let $M(t) \leq M$ be the upper bound (i.e., the size constraint) for the number of k-mers kept for each table at each node (i.e., AdaptiveSizeConstraint in Algorithm 1). Let $r : \mathcal{T} \to (0, 1]$ assign a portion of the full budget M to each node t; any function r is valid as long as it takes the value 1 at the root (i.e., $r(\text{root}) = 1$) and increases as we move up the tree (i.e., $r(t) \geq r(t')$ if $t' \in \text{child}(t)$). We evaluated two options for $r\,(t)$:

$$\begin{aligned} r\,(t) &= \frac{\sum_{s \in \mathcal{S}_t} |\mathcal{K}_s|}{\sum_{s \in \mathcal{S}} |\mathcal{K}_s|} \quad \text{(total } k\text{-mer count constraint), or} \\ r\,(t) &= \frac{|\mathcal{S}_t|}{|\mathcal{S}|} \qquad\qquad \text{(number of species constraint).} \end{aligned} \tag{7}$$

The two options for $r\,(t)$ would be identical if all species have equal-sized genomes and each species is sampled the same number of times ($\mathcal{K}_s$ can be the union over multiple genomes). Their main difference is that the first option allows highly sampled species (or very large genomes) to contribute more, while the latter does not. With either definition, we then set

$$M\,(t) = \begin{cases} |\mathcal{K}_t| & t \in \mathcal{S}, \\ \sqrt{r\,(t)}M & \text{otherwise} \end{cases} \tag{8}$$

noting that $M(t) \leq \sum_{t' \in \text{child}(t)} M(t')$ due to the concavity of the square root. The concavity matters because it ensures that the size constraint becomes more restrictive as we move up the tree. In this case, the allowed budget increases faster than proportionally until $r(t) = 1/\sqrt{2} \approx 0.7$ and slower than proportionally after. Thus, at lower ranks, many of the decisions for removing k-mers are left to its ancestors; note that a k-mer not removed at a taxon can still be removed further up. Close to the leaves, very few k-mers would be removed (e.g., a single species out of 10^4 still is assigned 1% of the total budget when visiting this node), while close to the root, taxon budgets get closer to the full budget (e.g., a phyla with $2/3$ of the species is assigned 82% of the budget). Also, going up the tree, the k-mer budget assigned to each taxon increases compared to each child, but it never exceeds the total budget assigned to its children. Clearly, $M(\text{root})$ matches the full budget allowed.

FILTERBYRANK

For each hash taxon t, the FILTERBYRANK function of Algorithm 1 removes k-mers when the size of the table $\mathbf{H}_t$ exceeds $M(t)$. First, each row is pruned to b k-mers according to the strategy described below. If the table size is still greater than $M(t)$, we continue removing k-mers, one at a time from each row in the descending order of their sizes, and iterating until the size constraint is satisfied.

For a k-mer set $\mathcal{K}$, a taxonomy $\mathcal{T}$, and its corresponding species set $\mathcal{S}$, we define $\mathrm{R}, \mathrm{R}' : \mathcal{K} \times \mathcal{T} \to [0, |\mathcal{S}|]$ as

$$\mathrm{R}(x,t) = |\{y \ : \ x \in \mathcal{K}_y,\, y \in \mathcal{S}_t\}| \tag{9}$$

and

$$\mathrm{R}'(x,t) = \sum\nolimits_{t' \in \mathrm{child}(t)} \mathbb{1}\left\{|\mathrm{R}(x,t')| > 0\right\} \ , \tag{10}$$

both illustrated in Fig. S2. We express each ranking strategy using either function and removing low-ranked k-mers accordingly. Discriminative and common k-mer strategies simply rank k-mers inversely and proportionally (respectively) to R or R'. In the case of ties, which can happen frequently, especially near leaves, we simply break them randomly.

The goal of covering all species can be imposed as a complex set covering problem. Instead, in the interest of scalability, we opt for a simple approach using weighted sums. We set

$$\mathrm{R}^*(x,t) = \sum\nolimits_{t' \in \mathrm{child}(t)} w_{t'}(x) \cdot \mathrm{R}(x,t') \tag{11}$$

where $w_{t'}$ down-weights groups that are highly sampled among surviving k-mers (see Fig. S2). A natural choice of weights is $w_t = 1/|\mathbf{H}_t|$. However, because $|\mathbf{H}_t|$ is independent of the k-mer or the row being considered, this approach will consistently down-weight the children with the largest tables; in the extreme case, it can remove all k-mers from a child altogether. Thus, we define the weight of a k-mer x locally for its LSH table row by setting it to be inversely proportional to the *coverage* of each child taxon among k-mers of that row:

$$w_{t'}(x) = \left(|\left\{x' \ : \ x' \in \mathbf{H}_{t'},\, \mathrm{LSH}(x) = \mathrm{LSH}(x')\right\}|\right)^{-1}$$

As a result, children covered with fewer *surviving* k-mers get higher weights, leading the process at the parent node to remove them less often (see Fig. S2 for a toy example). However, since decisions are made per row, and assignment of k-mers to rows is random, this avoids consistently removing the same species.

EXTRACTKMERS

In extracting k-mers from all input genomes, we use minimizers to perform an initial subsampling by choosing the k-mer whose encoding has a MurmurHash3 (Appleby, 2009) value that is the smallest in a local window of size $w = k + 3$. We adopt the left/right encoding of k-mers introduced by Rachtman, Bafna, et al. (2021); this encoding allows fast calculation of Hamming distance using just four instructions (a `pop-count`, an XOR, an OR, and a shift). For $k > 16$, these encodings require 64 bits. However, h many k-mer positions are already given by the hash index and do not need to be saved as a part of the encoding. In KRANK, unlike CONSULT-II, we save memory by dropping these positions; this reduces the encoding size to a 32bit number when $k - h \geq 16$, which is the case in our default configurations ($k = 30$ and $h = 14$ or $k = 29$ and $h = 13$). Since each k-mer encoding is only 32 bits, we save the encodings in the hash table; note that this is different from CONSULT-II, which saved pointers to a separate *encoding* array.

Populating LSH tables (FILLTABLE, UNIONTABLES, and PROJECTTABLE)

For each leaf t, FILLTABLE computes LSH and encoding values of each k-mer $x \in \mathcal{K}_t$ based on h positions, and stores k-mer's compact encoding in the row indexed by the LSH value. We keep a counter initialized to 1 associated with each k-mer to enable computing $\mathrm{R}(x, t)$ later. For non-leaf taxa, UNIONTABLES combines hash table rows by taking the union of k-mers. Luckily, the union operation can be defined on a per-row basis as the same k-mers share LSH value; independent union operations are performed in parallel across rows. Union also updates $\mathrm{R}(x, t)$. Furthermore, KRANK uses some of the available threads to perform post-order traversal in parallel.

Below root, KRANK saves rows of the hash tables using vectors that allow dynamic size changes during construction, switching to static structures only at the end. At the root, each row gets pruned to b k-mers, again using the ranking. This allows converting the table to a memory-efficient flattened static 1D array of size $2^{2h}b$.

Batching

Since we need to merge multiple tables at internal nodes, the required memory may exceed what is available. To tackle this issue, we used a batching approach. Except for the ADAPTIVESIZECONSTRAINT, all components of our algorithm can be performed independently on a per-row basis. Therefore, we divide rows of the tables into batches and build them separately and in parallel. KRANK has a parameter to set the number of batches, and each batch's size constraint is simply updated as $M(t)$ divided by the number of batches.

Datasets

WoL-v1 dataset (read classification)

We demonstrate the pros and cons of selection strategies using the microbial WoL-v1 dataset (Zhu et al., 2019) composed of 10,575 genomes. We excluded 100 archaeal genomes used by Rachtman, Bafna, et al. (2021) as queries and five genomes with IDs missing from NCBI from the reference set, leaving us with 569 archaeal and 9901 bacterial references. We chose 756 queries in total; these include 666 bacterial genomes added to RefSeq after WoL-v1 was constructed, 10 randomly selected bacteria from the reference set, and all 80 of 100 archaeal queries from Rachtman, Bafna, et al. (2021) with Jaccard index above 0 to some reference genome. The queries span a range of distances to the closest reference genome and are binned into novelty groups as such (Fig. S1A). The query set covers 55 phyla and 396 genera, with some phyla highly represented (e.g., 253 from *Pseudomonadota*, 91 from *Bacillota*) but including several rare phyla (Fig. S1B). We generated 150bp reads using ART (Huang et al., 2012) at high coverage with default Illumina error profile and then subsampled down to 66667 reads for each query ($\approx 2\times$ coverage for these genomes). We use the NCBI taxonomy provided with the WoL-v1 dataset as our reference taxonomy.

We used this dataset to explore the various heuristics of KRANK and to also compare final KRANK versus other methods. We examined the same query set in both analyses. We compared KRANK against CONSULT-II v0.4.0 (Şapcı et al., 2024), where k-mer selection is arbitrary, Kraken-II (with default settings), and CLARK (Ounit, Wanamaker, et al., 2015) (with default settings, k=31; species rank), which uses discriminative k-mers. For KRANK, the classification algorithm is identical to CONSULT-II v 0.5.0. We tested KRANK with two memory levels: KRANK-hs (high-sensitivity) sets $k = 30$, $h = 14$, $b = 16$ and uses 51.2Gb of memory while KRANK-lw (lightweight) sets $k = 29$, $h = 13$, $b = 16$ and 12.8Gb of memory. For details, see the exact commands and tool versions in the supplementary information.

Profiling on CAMI-I high complexity dataset

This dataset contains five different high-complexity samples, each of size 75Gbp, which are simulated by mimicking the abundance distribution of the underlying microbial communities (Sczyrba et

al., 2017). We tested the Bracken (Lu et al., 2017) extension of Kraken-II which combines its results with Bayesian priors for better profiling. We used the same custom libraries built with the WoL-v1 dataset, with default parameters as described in WoL-v1 dataset (read classification). Results for CONSULT-II, CLARK, and Bracken are retrieved from our prior work and are available at https://github.com/bo1929/shared.CONSULT-II.

Profiling on CAMI-II marine and strain-madness datasets

We used the ten-sample marine dataset (5Gbp each) and the 100-sample (2Gbp each) strain-madness dataset, which are the two main datasets focused on abundance profiling in the CAMI-II challenge. For all tools except CONSULT-II and KRANK, we used CAMI-II results submitted to the challenge available at https://github.com/CAMI-challenge/second_challenge_evaluation. We exclude versions of the methods that were submitted to the first challenge. CONSULT-II results were again retrieved from https://github.com/bo1929/shared.CONSULT-II. We built and used a KRANK library in high-sensitivity setting ($k = 30$, $h = 14$, $b = 16$) using 72,766 genomes selected from NCBI RefSeq snapshot as of 2019/01/08 by allowing each species to contribute at most 500 genomes (otherwise randomly selected among available genomes).

Evaluation metrics

We describe evaluation metrics, referring the reader to Supplementary material for further commands and details.

Read classification

We evaluated the read classification with respect to the NCBI taxonomy. Each prediction is separately evaluated across each taxonomic rank. When the reference set had at least one genome matching the query taxon at a particular rank, we call it a *positive*: TP if it is the correct taxon, FP if it is the incorrect taxon, and FN if the read is not classified at that rank. Similarly, at a particular rank, when the reference set lacks a genome from the query taxon, we call it a *negative*: TN if the read is not classified at that rank, FP if it is classified, which would necessarily be false. We ignored queries at ranks where the true taxonomic ID given by NCBI is a missing rank.

We use custom scripts to process the specific output format of each tool and compare it with the taxonomy used to build reference libraries. These scripts are available on https://github.com/bo1929/shared.KRANK. We also provide distances of each query genome to the closest reference genome d^* as estimated by Mash (Ondov et al., 2016), taxonomy files, and the outputs of evaluation scripts.

Taxonomic profiling

To measure taxonomic profiling performances on both CAMII challenges, we reported the same metrics as those emphasized in original publications. We used OPAL (version `1.0.12`) to compute the metrics with `-n` option (which renormalizes after removing the unclassified portion).

On CAMI-I, we used the main two metrics singled out by the open-community profiling assessment tool (OPAL) (Meyer, Bremges, et al., 2019): the Bray-Curtis dissimilarity between the estimated profile and the true profile and Shannon's equitability as a measure of alpha diversity. All the results are averaged across all 5 samples. On the CAMI-II Challenge, we followed the original paper by Meyer, Fritz, et al. (2022) and compared methods based on L1 norm error and weighted UniFrac error. Both L1 norm error and weighted UniFrac error measure the accuracy of the relative abundance estimations of taxa in a sample. The UniFrac metric is computed across the entire taxonomic tree, while the L1 norm is computed at each taxonomic rank. The weighted UniFrac has a maximum of 16 on this dataset, so we report 16 - UniFrac; similarly, the L1 norm can never be more than 2, so we report 2 - L1. We report averages across 10 samples and 100 samples respectively for marine and strain-madness datasets.

DATA ACCESS

WoL-v1 dataset was taken from https://biocore.github.io/wol/download. Simulated query sequences for read classification can be found at https://ter-trees.ucsd.edu/data/consult/KRANK-queries.tar.gz. CAMI-II marine and strain-madness datasets were taken from from https://frl.publisso.de/data/frl:6425521/marine/ and https://frl.publisso.de/data/frl:6425521/marine/ and https://frl.publisso.de/data/frl:6425521/strain/, respectively. CAMI-I high-complexity dataset was taken from http://gigadb.org/dataset/100344. The RefSeq snapshot and the corresponding taxonomy used for the library construction were retrieved from https://openstack.cebitec.uni-bielefeld.de:8080/swift/v1/CAMI_2_DATABASES/. CAMI-II results and ground truth profiles were retrieved from https://github.com/CAMI-challenge/second_challenge_evaluation. OPAL software and ground truth profiles for CAMI-I are available on GitHub: https://github.com/CAMI-challenge/OPAL. Our results, metadata files and scripts used to create figures are available at https://github.com/bo1929/shared.KRANK. KRANK's software can be found on GitHub https://github.com/bo1929/KRANK.

References

Appleby, A (2009). *MurmurHash3*.

Asnicar, F et al. (Dec. 2020). “Precise phylogenetic analysis of microbial isolates and genomes from metagenomes using PhyloPhlAn 3.0”. In: *Nature Communications* 11.1. Publisher: Springer US ISBN: 0348556977, p. 2500.

Balaban, M, Y Jiang, Q Zhu, D McDonald, R Knight, and S Mirarab (July 2023). “Generation of accurate, expandable phylogenomic trees with uDance”. In: *Nature Biotechnology* Online.

Har-Peled, S, P Indyk, and R Motwani (2012). “Approximate nearest neighbors: Towards removing the curse of dimensionality”. In: *Theory of Computing* 8.1, pp. 321–350.

Huang, W, L Li, JR Myers, and GT Marth (2012). “ART: A next-generation sequencing read simulator”. In: *Bioinformatics* 28.4, pp. 593–594.

Lee, D, R Karchin, and MA Beer (Dec. 2011). “Discriminative prediction of mammalian enhancers from DNA sequence”. en. In: *Genome Research* 21.12, pp. 2167–2180.

Li, H (2018). *Seqtk, toolkit for processing sequences in FASTA/Q formats.*

Liang, Q, PW Bible, Y Liu, B Zou, and L Wei (Mar. 2020). “DeepMicrobes: taxonomic classification for metagenomics with deep learning”. In: *NAR Genomics and Bioinformatics* 2.1.

Lu, J, FP Breitwieser, P Thielen, and SL Salzberg (Jan. 2017). “Bracken: estimating species abundance in metagenomics data”. In: *PeerJ Computer Science* 3, e104.

Marçais, G and C Kingsford (2011). “A fast, lock-free approach for efficient parallel counting of occurrences of k-mers”. In: *Bioinformatics* 27.6, pp. 764–770.

McDonald, D et al. (July 2023). “Greengenes2 unifies microbial data in a single reference tree”. In: *Nature Biotechnology* Online.

Meijenfeldt, FAB von, K Arkhipova, DD Cambuy, FH Coutinho, and BE Dutilh (Dec. 2019). “Robust taxonomic classification of uncharted microbial sequences and bins with CAT and BAT”. In: *Genome Biology* 20.1, p. 217.

Meyer, F, A Bremges, P Belmann, S Janssen, AC McHardy, and D Koslicki (2019). “Assessing taxonomic metagenome profilers with OPAL”. In: *Genome Biology* 20.1, p. 51.

Meyer, F, A Fritz, et al. (Apr. 2022). “Critical Assessment of Metagenome Interpretation: the second round of challenges”. en. In: *Nature Methods* 19.4. Number: 4 Publisher: Nature Publishing Group, pp. 429–440.

Nasko, DJ, S Koren, AM Phillippy, and TJ Treangen (Dec. 2018). “RefSeq database growth influences the accuracy of k-mer-based lowest common ancestor species identification”. In: *Genome Biology* 19.1, p. 165.

Ondov, BD, TJ Treangen, P Melsted, AB Mallonee, NH Bergman, S Koren, and AM Phillippy (Dec. 2016). “Mash: fast genome and metagenome distance estimation using MinHash”. In: *Genome Biology* 17.1, p. 132.

Ounit, R and S Lonardi (Dec. 2016). “Higher classification sensitivity of short metagenomic reads with CLARK-S.” In: *Bioinformatics (Oxford, England)* 32.24, pp. 3823–3825.

Ounit, R, S Wanamaker, TJ Close, and S Lonardi (Dec. 2015). “CLARK: fast and accurate classification of metagenomic and genomic sequences using discriminative k-mers”. In: *BMC Genomics* 16.1, p. 236.

Pachiadaki, MG et al. (2019). “Charting the Complexity of the Marine Microbiome through Single-Cell Genomics”. In: *Cell* 179.7, 1623–1635.e11.

Parks, DH, M Chuvochina, DW Waite, C Rinke, A Skarshewski, PA Chaumeil, and P Hugenholtz (Nov. 2018). “A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life”. In: *Nature Biotechnology* 36.10, pp. 996–1004.

Rachtman, E, V Bafna, and S Mirarab (June 2021). “CONSULT: accurate contamination removal using locality-sensitive hashing”. In: *NAR Genomics and Bioinformatics* 3.3, pp. 10.1101/2021.03.18.436035.

Rachtman, E, M Balaban, V Bafna, and S Mirarab (May 2020). “The impact of contaminants on the accuracy of genome skimming and the effectiveness of exclusion read filters”. In: *Molecular Ecology Resources* 20.3, pp. 1755–0998.13135.

Roberts, M, W Hayes, BR Hunt, SM Mount, and JA Yorke (Dec. 2004). “Reducing storage requirements for biological sequence comparison.” In: *Bioinformatics (Oxford, England)* 20.18, pp. 3363–3369.

Şapcı, AOB, E Rachtman, and S Mirarab (2024). “CONSULT-II: Accurate taxonomic identification and profiling using locality-sensitive hashing”. en. In: *Bioinformatics (in press)*.

Sczyrba, A et al. (Nov. 2017). “Critical Assessment of Metagenome Interpretation—a benchmark of metagenomics software”. In: *Nature Methods* 14.11, pp. 1063–1071.

Wood, DE, J Lu, and B Langmead (Dec. 2019). “Improved metagenomic analysis with Kraken 2”. In: *Genome Biology* 20.1, p. 257.

Zheng, H, G Marçais, and C Kingsford (Aug. 2023). “Creating and Using Minimizer Sketches in Computational Genomics”. en. In: *Journal of Computational Biology*, cmb.2023.0094.

Zhu, Q et al. (2019). “Phylogenomics of 10,575 genomes reveals evolutionary proximity between domains Bacteria and Archaea”. In: *Nature Communications* 10.1, p. 5477.

Supplementary Information

Supplementary Methods

Evaluation metrics and tools used

To compute recall, precision, and F1 for read classification, we used the following commands, which can be run after setting the current working directory to https://github.com/bo1929/shared.KRANK:

1. `python scripts/evaluate.py -output-dir $OUTPUT_DIR \`
   `-taxonomy-database-path data/ReferenceTaxonomy-nodes.dmp -tvth 0.03 \`
   `-reference-ranks-path data/10kBacteria-ranks_tid.tsv \`
   `-query-ranks-path data/uDance-ranks_tid.tsv -results-path $RESULTS_FILE`
   where `$RESULTS_FILE` is the output report of CONSULT-II, including the prediction for each read, and `$OUTPUT_DIR` is simply the output directory,
2. `python scripts/summarize_evaluations.py $OUTPUT_FILE \`
   `data/dist-to-closest.txt 1 > $EVALUATION_SUMMARY`
   where `$OUTPUT_FILE` is the output of the previous command, and `$EVALUATION_SUMMARY` is the summary results across all reads used to generate figures.

For the CAMI-II challenge, we ran the commands given at https://github.com/CAMI-challenge/second_challenge_evaluation/tree/master/profiling, using the results of all participants provided in the same repository. For CAMI-I, we used the ground-truth given at https://github.com/CAMI-challenge/OPAL/blob/master/data/gs_cami_i_hc.profile. To post-process abundance estimates of each tool to the CAMI profiling Bioboxes format, we used scripts found at https://github.com/CAMI-challenge/docker_profiling_tools.

Parameter configurations of tools

For CONSULT-II, we used l=2 and k=32 (minimized from canonical 35-mers) for all memory levels. We set hash table parameters h and b to `-h 15 -b 7`, `-h 13 -b 14`, `-h 12 -b 16`, and `-h 12 -b 9`, respectively for 140.7Gb, 16Gb, 5Gb and 3Gb libraries. For other parameters (LCA probability function and maximum match distance), default values were used.

As Rachtman, Balaban, et al. (2020) found default settings to be preferable for query identification, we used default settings for Kraken-II (`-kmer-len 35`, `-minimizer-len 31`, and `-minimizer-spaces 7`) and CLARK (k=31, default classification mode, species rank for classification, and `-m 1`). We used `-max-db-size` parameter to adjust Kraken-II database sizes (4Gb, 16Gb, and default 46Gb).

For KRANK, we used the following parameter configurations for corresponding library sizes; 76.8Gb: `-k 30 -w 33 -h 14 -b 16` with $l = 3$, 51.2Gb: `-k 30 -w 33 -h 14 -b 16` with $l = 2$ (high-sensitivity), 25.6Gb: `-k 30 -w 33 -h 14 -b 16` with $l = 1$, 12.8Gb: `-k 29 -w 32 -h 13 -b 16` with $l = 2$ (lightweight), 6.4Gb: `-k 29 -w 32 -h 13 -b 16` with $l = 1$, 3.2Gb: `-k 28 -w 31 -h 12 -b 16` with $l = 2$, 1.6Gb: `-k 28 -w 31 -h 12 -b 16` with $l = 1$.

Software versions and commands used

Here, we provide the exact commands that we used to run external tools, CONSULT-II, and KRANK throughout our experiments, together with their version information.

Genomic distance estimation using Mash (Ondov et al., 2016)

We used Mash (version `2.3`) to estimate genomic distances. To create a Mash sketch from a genome and then use it to estimate genomic distance, we used the below commands.

1. `mash sketch -k 30 -s 750000 -p $NTHREADS -o $SKETCH_FILE $INPUT_FASTA`

2. `mash dist $SKETCH_FILE1 $SKETCH_FILE2`

Short read simulation with reference using ART (Huang et al., 2012)

We simulated short reads with length L=150 and coverage c, with the default error and quality profiles of Illumina HiSeq 2500 using ART (version `2.5.8` – single read mode) with the command below.

```
art_illumina -ss HS25 -l 150 -f c -na -s 10 -i $INPUT_FASTA -o $OUTPUT_FASTQ
```

Extraction of k-mer sets using Jellyfish (Marçais and Kingsford, 2011)

We used Jellyfish (`v2.3.0`) to compute distinct k-mer sets and k-mer profiles (for canonical 32-mers and 35-mers) of concatenated reference genomes (in FASTA format). The command is given below.

```
jellyfish count -m 35 -s 100M -t 24 -C $INPUT_FASTA -o $COUNT_FILE
```

Then, to output the list of distinct k-mers together with their counts into a FASTA file, we used the following command. Note that we do not use count values.

```
jellyfish dump $COUNTS_FILE > $OUTPUT_FASTA
```

Downsampling reads with `seqtk` (Li, 2018)

To subsample read collection down to a specified number of reads, denoted by n here, we used `seqtk` (version `1.3r106`) with the command below.

```
seqtk sample -s150 INPUT_FASTQ n > OUTPUT_FASTQ
```

Building a KRANK library and querying sequences

The version of KRANK we presented in this paper is `v0.4.0`. Running the following commands in order creates a KRANK library that can be queried against to find k-mer matches.

1. We first initialize a library, and preprocess all input genomes to extract k-mers. We built two libraries to use together: one with `-adaptive-size` option and the other with `-free-size` option, random seeds set to 0 and 19, respectively. We set k-mer length (`-k`) to 30, minimizer window size (`-w`) to 33, number of LSH positions (`-h`) to 14, number of table columns (`-b`) to 16, and number of bits to determine batch IDs (`-s`) to 9 (hence 2^9 batches in total). Two files are required to be given with arguments (`-t` and `-i`): `$TAXONOMY_NODES` is an NCBI taxonomy `nodes.dmp` file and `$INPUT_MAP_FILE` is a tab-separated file, its first column is for the species IDs and the second column is for the genome paths. The option `-input-sequences` specifies that input files are genomes, not k-mers extracted by some external tool, `-from-scratch` specifies that the library is to be initialized.

```
krank -seed $RANDOM_SEED build -k 30 -w 33 -h 14 -b 16 -s 9 \
-l $LIBRARY_DIR -t $TAXONOMY_NODES -i $INPUT_MAP_FILE \
-kmer-ranking 1 -adaptive-size \
-from-scratch -input-sequences -num-threads $NTHREADS
```

2. Once the library is initialized, each batch can be built separately (i.e., `-target-batch` 1 to 512) using the below command. Note that this command also adds the soft-LCA information to the library.

```
krank -seed $RANDOM_SEED build \
-l $LIBRARY_DIR -t $TAXONOMY_NODES -i $INPUT_MAP_FILE \
-kmer-ranking 1 -adaptive-size -from-library -num-threads $NTHREADS \
-target-batch $BATCH_NUM -input-sequences
```

3. Lastly, we queried sequences against the constructed library and generated a CONSULT-II compatible k-mer match information file for further classification and profiling.

```
krank query -l $LIBRARY_DIR -o $OUTPUT_DIR -q $QUERY_PATHS_FILE \
-save-match-info -max-match-distance 5 -num-threads $NTHREADS
```

Taxonomic identification with Kraken-II (Wood et al., 2019) and Bracken (Lu et al., 2017)

We used `v2.1.3` and `v2.8` for Kraken-II and Bracken in our experiments with their default parameters, respectively. For CAMI-II (Meyer, Bremges, et al., 2019), the presented results are for Bracken `v2.2`, which was the version submitted to the challenge.

In order to construct a custom Kraken-II reference library from WoL-v1 genomes, and to extend it with Bracken for abundance profiling, we used the following commands:

1. We first initialized a library named `$DBNAME`.
   ```
   kraken2-build -download-taxonomy -db $DBNAME
   ```
2. Next, we changed the downloaded taxonomy to match the taxonomy files provided with the WoL-v1 database, retrieved from https://biocore.github.io/wol/data/taxonomy/.
3. We then added our custom genomes to the reference database.
   ```
   find $REFERENCE_GENOMES_DIR -name '*.fna' -print0 | \
   xargs -0 -I -t -n1 kraken2/kraken2-build -add-to-library  -db $DBNAME
   ```
4. After that, we built the database with specified k-mer & minimizer lengths `$KMER_LEN` (35) & `$MINIMIZER_LEN` (31) and default number of wind-carding positions $s = 7$.
   ```
   kraken2-build -build -db $DBNAME -threads 14 -max-db-size $MAX_SIZE \
   -kmer-len $KMER_LEN -minimizer-len $MINIMIZER_LEN -minimizer-spaces s
   ```
5. Lastly, given read length `$READ_LEN` (150), we processed the constructed library with Bracken.
   ```
   ./bracken-build -d $DBNAME -t $NTHREADS -k $KMER_LEN -l $READ_LEN
   ```

To query against the Kraken-II reference library for read classification and Bracken for abundance profiling, we used the commands below.

1. ```
   kraken2 -use-names -threads $NTHREADS -report $REPORT_FILE \
   -db $DBNAME $QUERY_FASTQ > $KRAKEN_OUTPUT
   ```
2. ```
   bracken -d $DBNAME -i $KRAKEN_OUTPUT -o $BRACKEN_OUTPUT \
   -r $READ_LEN -l S -t $NTHREADS
   ```

Taxonomic identification with CLARK (Ounit, Wanamaker, et al., 2015)

We used CLARK `v1.2.6.1` with its default parameters. CLARK failed to set target IDs for 494 genomes out of 10470 WoL-v1 genomes during the construction of its database. These genomes were added manually by setting their corresponding taxon.

We followed the below steps to build a custom CLARK database in the directory `$DIRDB`.

1. Create the directory `Custom/` inside `$DIRDB` directory.
2. Copy reference WoL-v1 genomes with accession numbers into the `Custom/` directory.
3. Similar to Kraken-II, we set the taxonomy to the one provided with the WoL-v1 database, retrieved from https://biocore.github.io/wol/data/taxonomy/.
4. We ran `./set_target.ssh $DIRDB custom -species`.

We queried sequences against the CLARK database using the default mode (i.e., `-m 1`), and then used these classification results to estimate abundances by running the commands below.

1. `./classify_metagenome.sh -O $QUERY_FASTQ -R $RESULT_FILE -n $NTHREADS -m 1`
2. `./estimate_abundance.sh -F $RESULT_FILE -D $DIRDB`

Taxonomic identification with CONSULT-II (Şapcı et al., 2024)

We used CONSULT-II version `v0.4.0` for all experiments except profiling with KRANK databases, for which we used version `v0.5.0`. Note that, `v0.5.0` was not used in the original manuscript of CONSULT-II and differs from `v0.4.0` mainly at species-level abundance profiling. For CAMI-II profiling, we directly used results presented in Şapcı et al. (2024) (for CONSULT-II) and Meyer, Fritz, et al. (2022) (for all other tools).

We used the commands below to build CONSULT-II reference libraries.

1. First, we minimized canonical reference 35-mers (output of `jellfish dump`) to 32-mer minimizers with a custom script (version `v0.3.0`), provided with CONSULT-II.
```
minimize -i $INPUT_35MERS_FASTA -o $OUTPUT_32MERS_FASTA
```
2. For the default 140.7Gb memory level, we used the command below to create the library. Here, `$INPUT_FASTA` is the output of above command: `$OUTPUT_32MERS_FASTA`. For 16Gb and 5Gb versions, one can set `-h 13 -b 14` and `-h 12 -b 16`, respectively.
```
consult_map -input-fasta-file $INPUT_FASTA -output-library-dir $LIBDIR \
-number-of-positions 15 -t 2 -l 2 -b 7.
```
3. After constructing the reference library, we added taxonomic information (soft-LCA information per k-mer) by using the following three commands:
   (a) Adding soft-LCAs requires an auxiliary file (`$TAXONOMY_LOOKUP`) that can be generated from a taxonomy file (`$TAXONOMY_NODES`, i.e., `nodes.dmp`), using a script CONSULT-II provides.
   ```
   python scripts/construct_taxonomy_lookup.py \
   -from-taxonomy-database $TAXONOMY_NODES
   ```
   (b) Then, running these two commands in order would finalize the library construction. Note that `$REFERENCE_GENOMES_DIR` is the directory in which all reference genomes are stored as FASTQ files.
   ```
   consult_search -i $LIBDIR -taxonomy-lookup-path $TAXONOMY_LOOKUP \
   -q $REFERENCE_GENOMES_DIR -filename-map-path $FILENAME_MAP \
   -thread-count $NTHREADS -init-ID
   consult_search -i $LIBDIR -taxonomy-lookup-path $TAXONOMY_LOOKUP \
   -q $REFERENCE_GENOMES_DIR -filename-map-path $FILENAME_MAP \
   -thread-count $NTHREADS -update-ID
   ```

Performing taxonomic classification and profiling contains two stages: finding k-mer matches and reporting them in an appropriate file format and then utilizing them to predict read-level assignments or to estimate sample-level composition estimates.

1. The below command outputs the match information files per query (`$MATCH_INFO`), with filenames that are query filenames prefixed with `match-info_*`, to `$OUTPUT_DIR`. This file format can also be generated by KRANK, and hence, the next steps are identical to CONSULT-II.
```
consult_search -i $LIBDIR -q $QUERY_FASTQ -o $OUTPUT_DIR \
-maximum-distance 5 -save-matches -thread-count $NTHREADS
```
2. To classify each read, simply run:
```
consult_classify -i $MATCH_INFO -o $OUTPUT_DIR \
-taxonomy-lookup-path $TAXONOMY_LOOKUP
```
3. For profiling, the below two commands are needed.
   (a) We first predict the number of reads belonging to each taxon.
   ```
   consult_profile -i $MATCH_INFO -o $OUTPUT_DIR \
   -taxonomy-lookup-path $TAXONOMY_LOOKUP
   ```
   (b) Then, using the output of the above command, we perform normalization and genome size correction. Here, `$THRESHOLD` (1000 in our case) is the total vote threshold for a taxon to be included, `$ABUNDANCE_REPORT` is the `consult_profile`'s output, `$NCBI_TAXONOMY_DIR` is a directory which stores `nodes.dmp` and `names.dmp` files, `$METADATA_FILE` is a tab seperated file which is required to contain `species_id` and `total_length` columns for each reference genome. The `total_length` corresponds to the genome size of the genome belonging to species `species_id`. `$S_ID` is a sample identifier name for the header of the report.
   ```
   python scripts/postprocess_profiles.py $THRESHOLD $ABUNDANCE_REPORT \
   $NCBI_TAXONOMY_DIR $METADATA_FILE $S_ID
   ```

Supplementary Figures

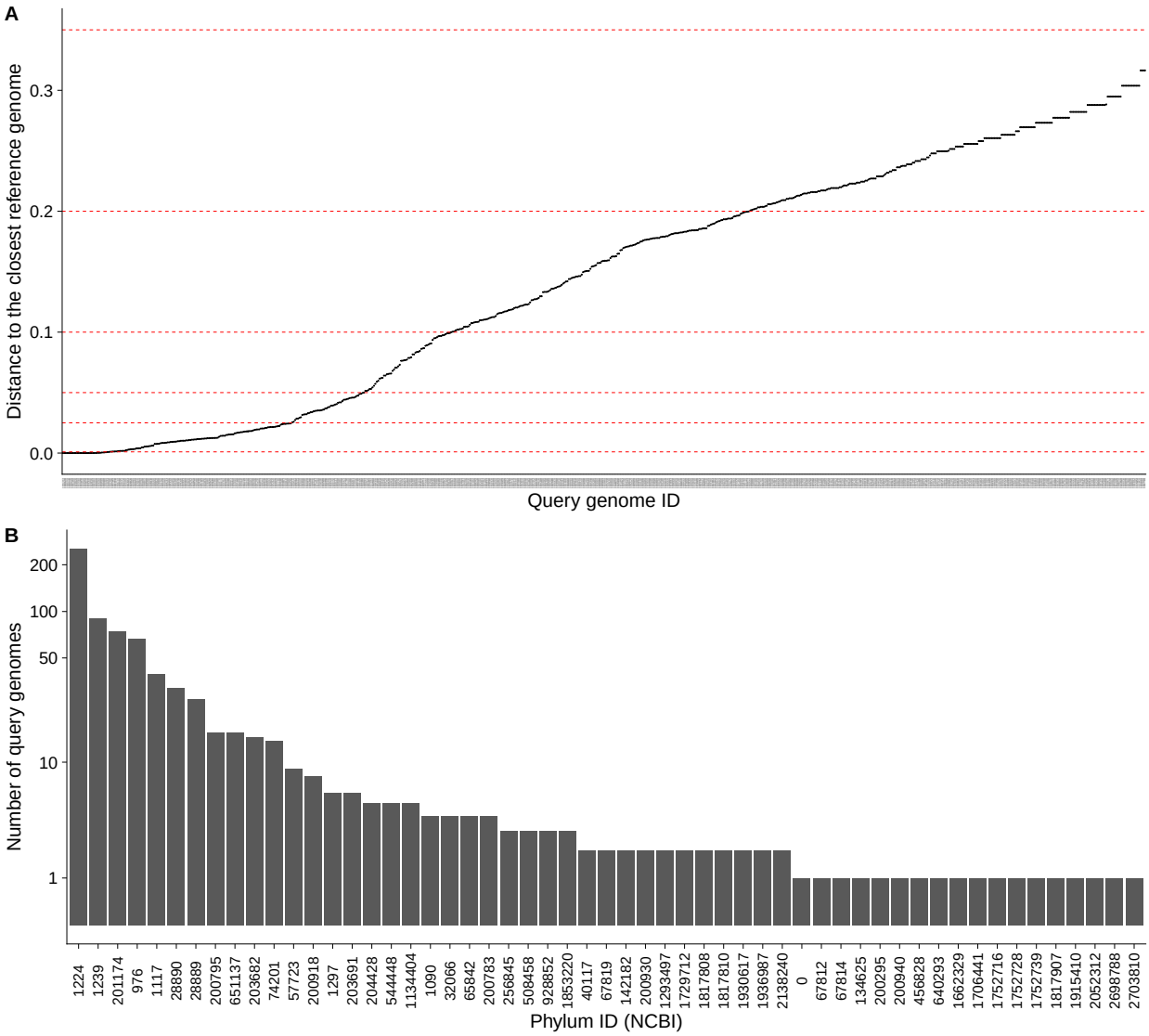


Figure S1: A) The distance to the closest reference genome d^*, as estimated by Mash, of each query genome. Each data point is for a query genome (x-axis). Dotted horizontal lines show boundaries of bins. B) Distribution of 765 queries across different phyla. The number of query genomes from the corresponding phylum is given the y-axis, and the x-axis is the NCBI ID of the phylum. 0 refers to genomes with unknown/unassigned groups at the phylum level. The y-axis is in one plus log scale.

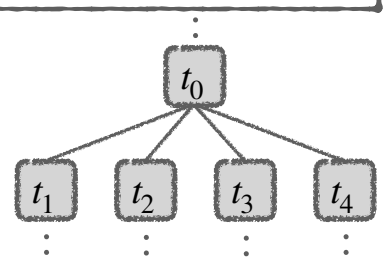
<u>An example set of rows indexed by the same LSH :</u> (k-mer x, species count $\mathrm{R}(x, t)$)

t_1 (ATTTCGGT,7),(CTTGGGTT,2),(ATTGGGTT,1),(ATTGCCTT,1)

t_2 (CCTTCGGT,3),(CTTGGGTT,3)

t_3 (CTTGGGTT,3),(ATTGGGTT,2)

t_4 (ATTGGGTT,2)



$\mathrm{R}(\texttt{ATTGCCTT}, t_0) = 1$	$\mathrm{R}'(\texttt{ATTGGGTT}, t_0) = 1$	$\mathrm{R}^*(\texttt{ATTGCCTT}, t_0) = 0.25$
$\mathrm{R}(\texttt{ATTGGGTT}, t_0) = 2$	$\mathrm{R}'(\texttt{CCTTCGGT}, t_0) = 1$	$\mathrm{R}^*(\texttt{ATTGGGTT}, t_0) = 1.25$
$\mathrm{R}(\texttt{ATTGGGTT}, t_0) = 3$	$\mathrm{R}'(\texttt{ATTGCCTT}, t_0) = 1$	$\mathrm{R}^*(\texttt{CCTTCGGT}, t_0) = 1.5$
$\mathrm{R}(\texttt{CCTTCGGT}, t_0) = 3$	$\mathrm{R}'(\texttt{ATTTCGGT}, t_0) = 1$	$\mathrm{R}^*(\texttt{ATTTCGGT}, t_0) = 1.75$
$\mathrm{R}(\texttt{ATTTCGGT}, t_0) = 7$	$\mathrm{R}'(\texttt{ATTGGGTT}, t_0) = 2$	$\mathrm{R}^*(\texttt{ATTGGGTT}, t_0) = 2.0$
$\mathrm{R}(\texttt{CTTGGGTT}, t_0) = 8$	$\mathrm{R}'(\texttt{CTTGGGTT}, t_0) = 3$	$\mathrm{R}^*(\texttt{CTTGGGTT}, t_0) = 3.5$

Figure S2: An illustration of a toy example to compare different k-mer ranking strategies: R, R', and R^*. Tables of sibling taxa t_1, t_2, t_3, and t_4 are being merged for parent t_0's table. For a particular LSH index, t_1, t_2, t_3, and t_4 have 4, 2, 2, and 1 k-mers in their corresponding row, respectively. Hence, weights for R^* will be $1/4$, $1/2$, $1/2$, and 1.

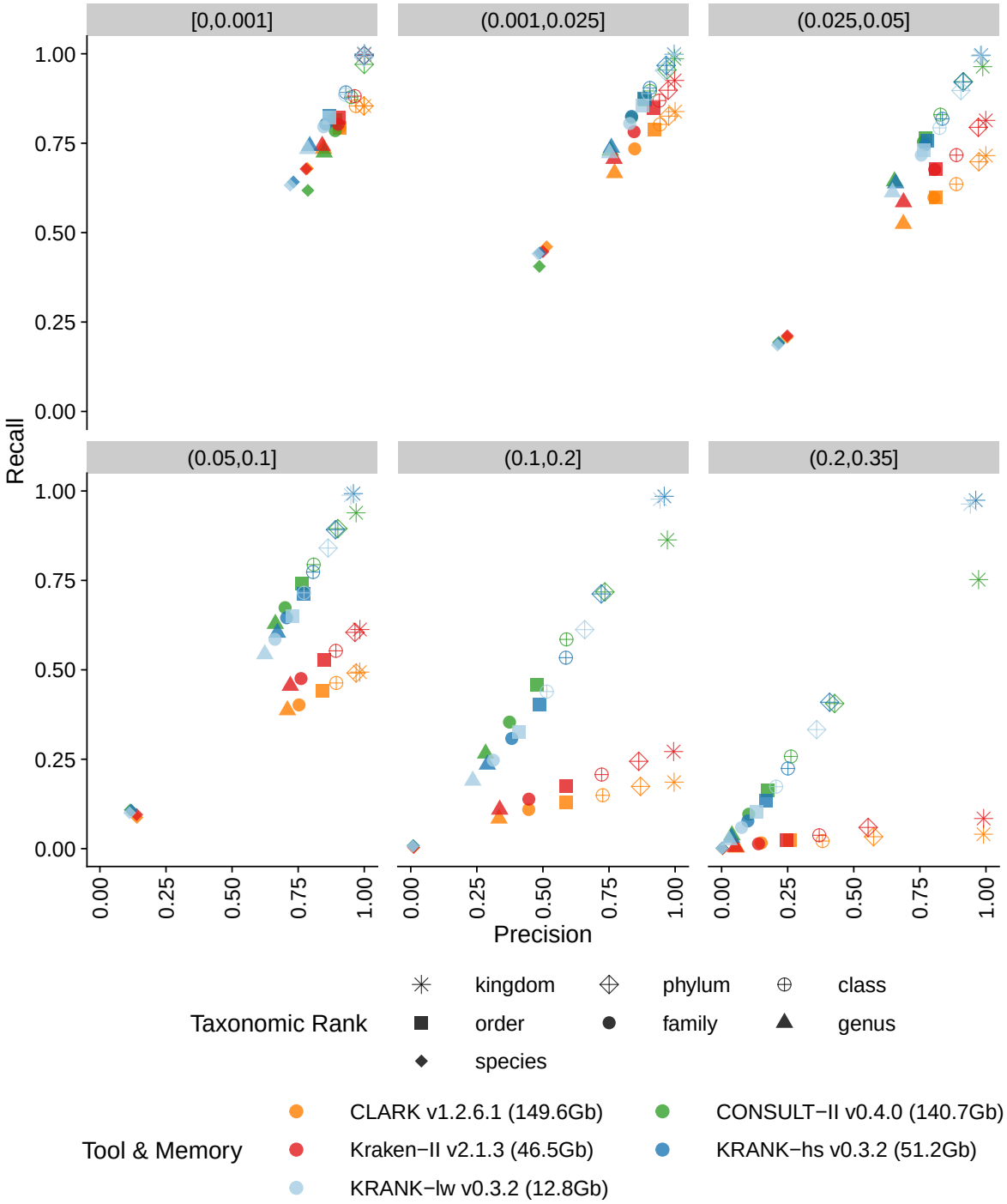


Figure S3: A more fine-grained comparison of the high-sensitivity (hs) and the lightweight (lw) modes of KRANK with other methods across different ranks on the WoL-v1 dataset with 66667 simulated 150bp reads from each of 756 query genomes. Results are averaged over all queries and reported per novelty bins (d^*; see Fig. S1) and ranks in terms of precision (x-axis) and recall (y-axis) scores.

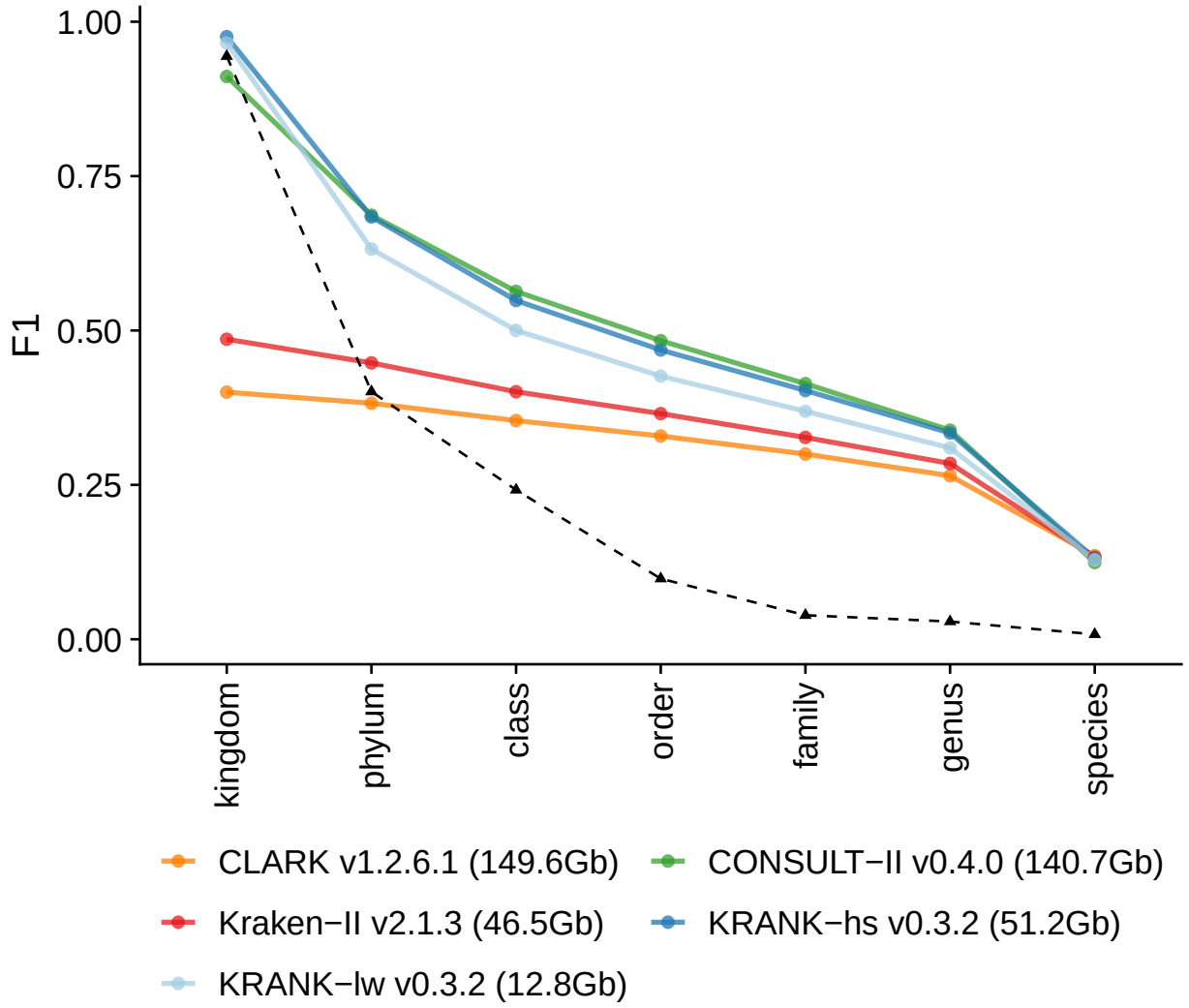


Figure S4: Comparison of KRANK with other methods across different ranks on the WoL-v1 dataset with 66667 simulated 150bp reads from each of 756 query genomes. Results are averaged over all queries. The dashed line stands for the *null model* which assigns all sequences to the largest taxon (in terms of number of query genomes) in the query set for the corresponding rank.

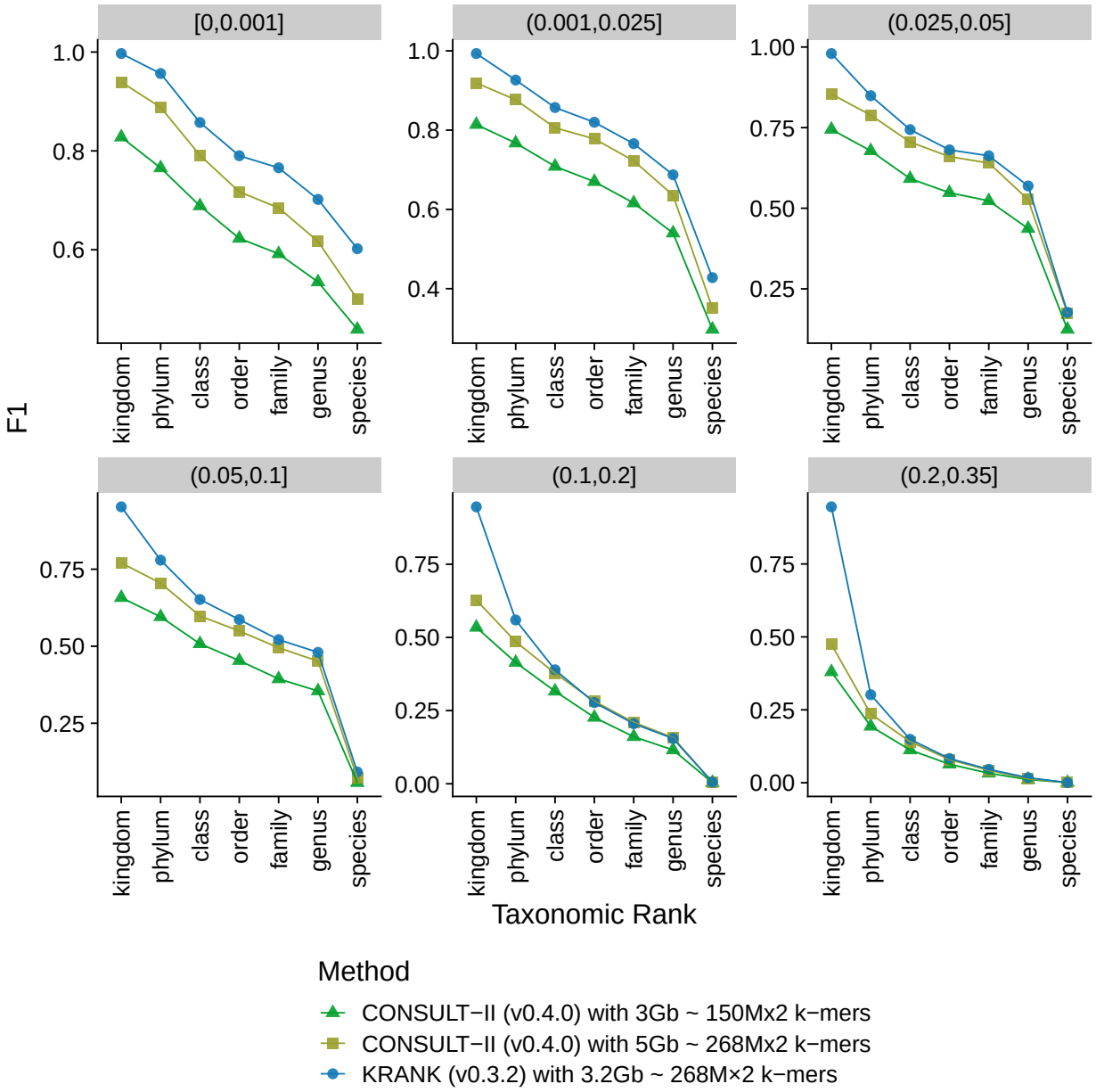


Figure S5: Comparison of CONSULT-II and KRANK in cases with the same memory level and the same number of k-mers in the library. KRANK can store 268M k-mer in a single table using less memory (1.8), but the selected k-mer set further improves the performance regardless of the memory. F1 scores are reported across all ranks as averages of all queries in each novelty bin.

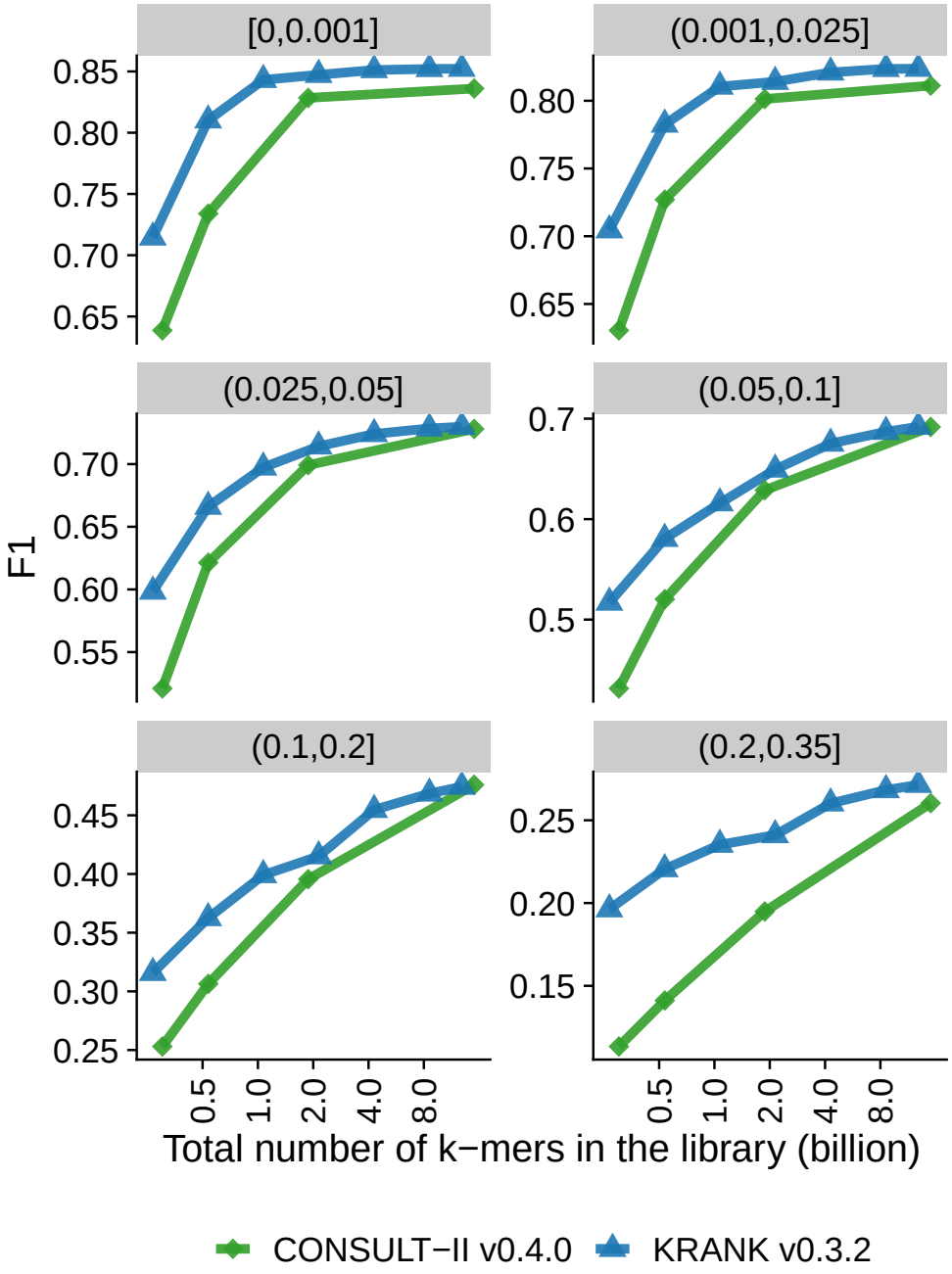


Figure S6: Comparison of classification performances CONSULT-II and KRANK as the total number of non-distinct k-mers kept in the library. Each panel is a novelty bin, corresponding to the minimum distance to any reference genome (d^*), measured by Mash (Ondov et al., 2016). F1 scores are reported across all ranks as averages of all queries in each novelty bin.

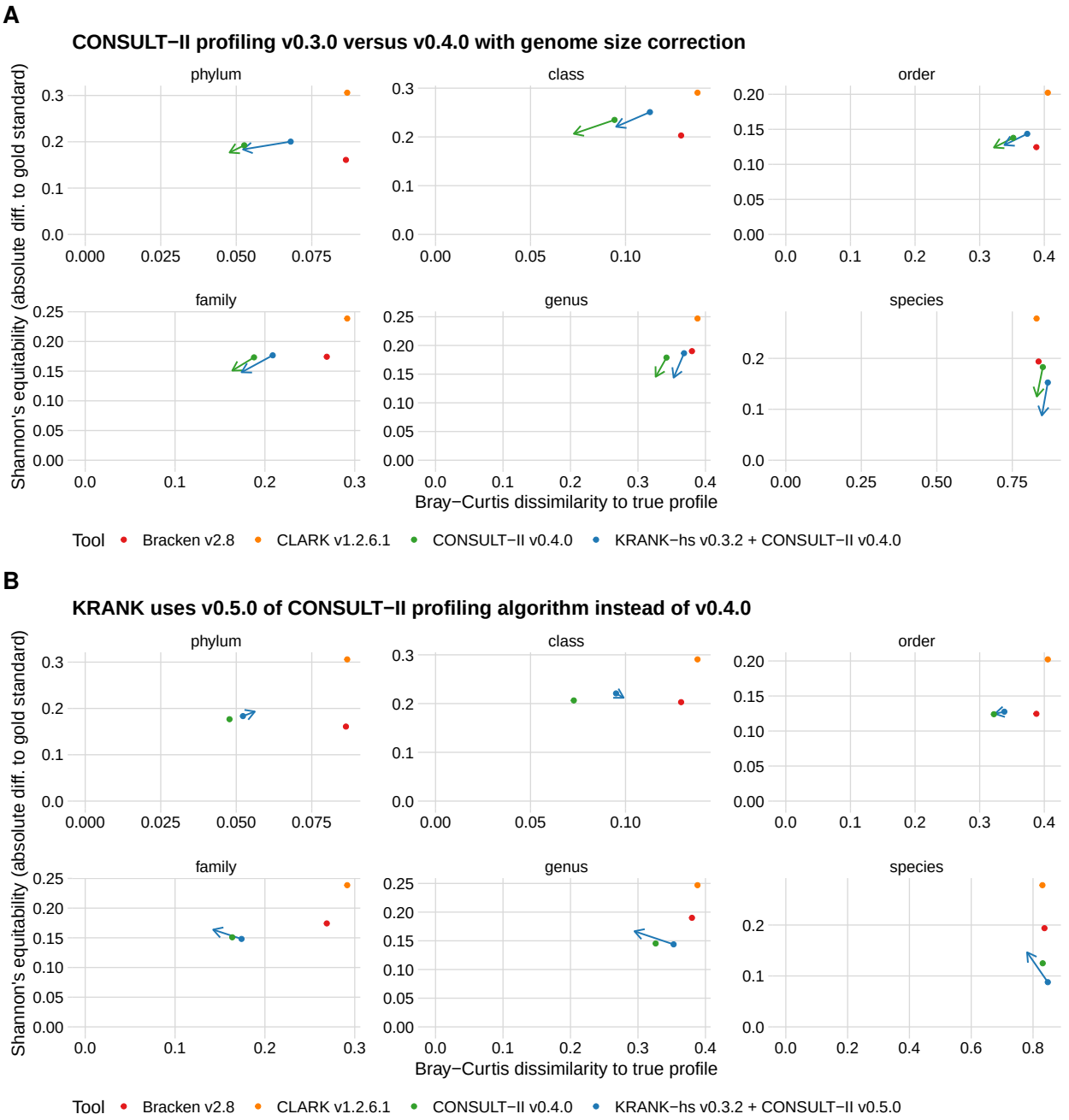


Figure S7: CONSULT-II and KRANK improvements across versions on CAMI-I dataset, based on Bray-Curtis dissimilarity to the true profile and Shannon's equitability difference with the gold standard. The bottom-left corner is perfect accuracy. A) CONSULT-II v0.4.0 versus v.0.3.0. The arrows show v0.3.0 (dot) and v0.4.0 (arrowhead) performance for KRANK and CONSULT-II. Applying genome size correction as a post-processing step in v0.4.0 improves v0.3.0. Note that, we use v0.4.0 for CONSULT-II profiling results, and v0.5.0 for KRANK profiling results. B) KRANK performance when using CONSULT-II v0.4.0 versus v0.5.0 for the profiling step. We use v0.5.0 and 0.4.0 throughout the manuscript respectively for KRANK and CONSULT-II profiling. We use v.0.4.0 for CONSULT-II because this version is used by Şapcı et al. (2024) and improvements of v0.5.0 were developed as part of this paper.

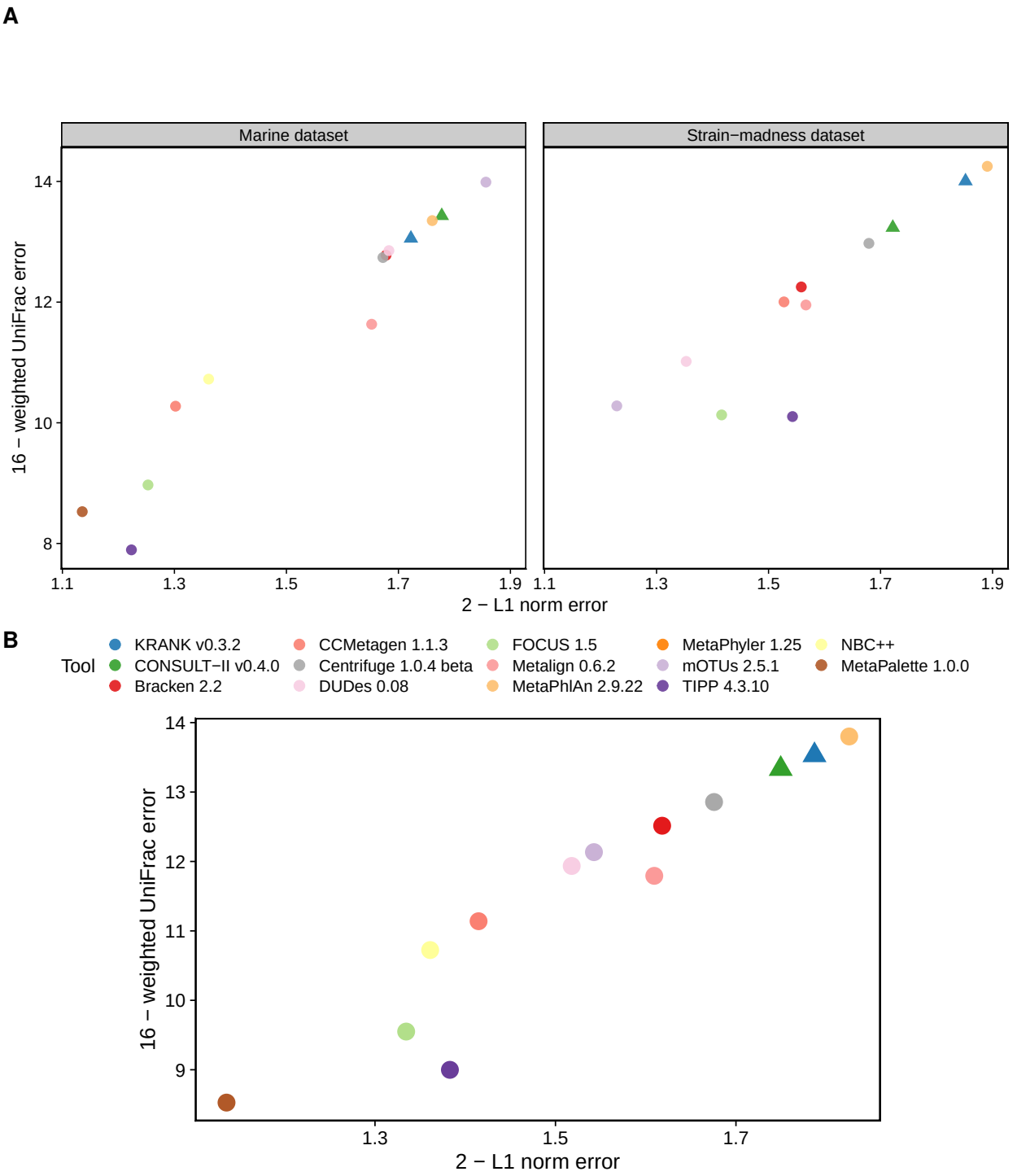


Figure S8: Comparing KRANK with other participants in CAMI-II benchmarking challenge (Meyer, Fritz, et al., 2022). We report average L1 norm error averaged across all ranks and the rank invariant weighted UniFrac error. A) Each data point stands for the average of 100 strain-madness samples or 10 marine samples. B) Each data point stands for the average of two datasets: the average of 100 strain-madness samples and the average of 10 marine samples. Metrics were computed using OPAL with default settings and the `-n` option.

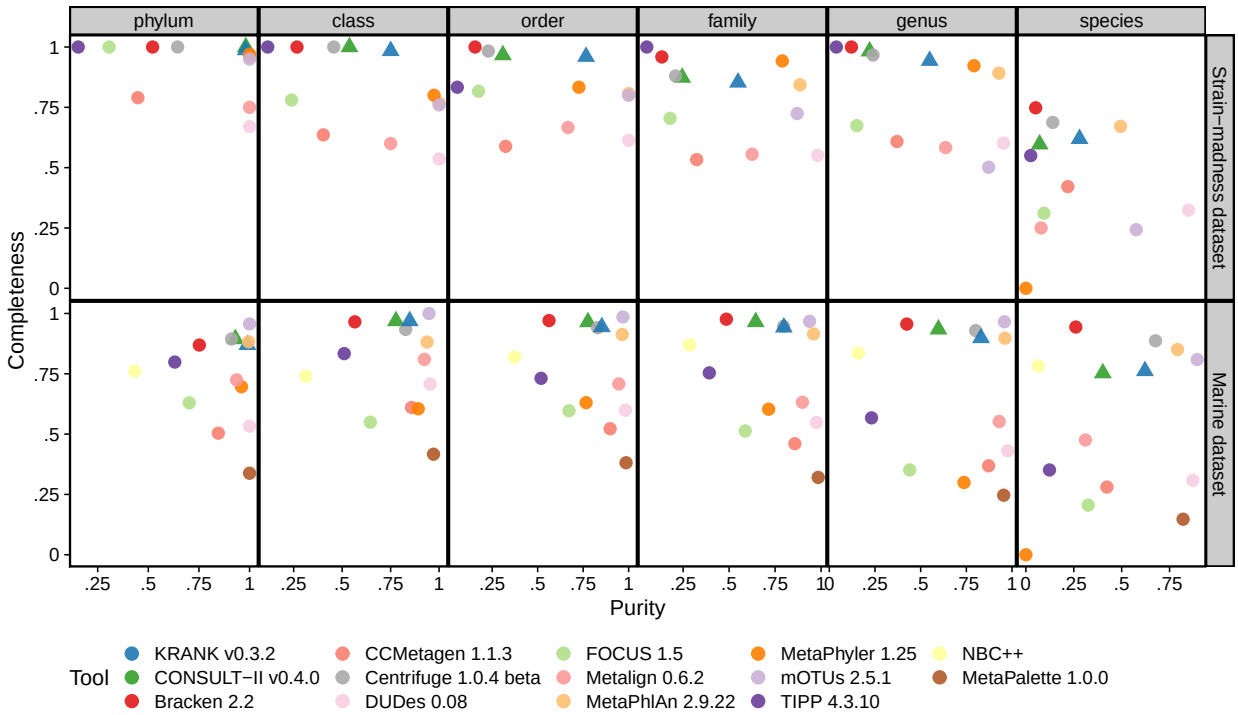


Figure S9: Comparing KRANK with other participants in CAMI-II benchmarking challenge (Meyer, Fritz, et al., 2022). As in the original manuscript, we show purity versus completeness. Each data point stands for the average of 100 strain-madness samples or 10 marine samples. Metrics were computed using OPAL with default settings and the `-n` option.

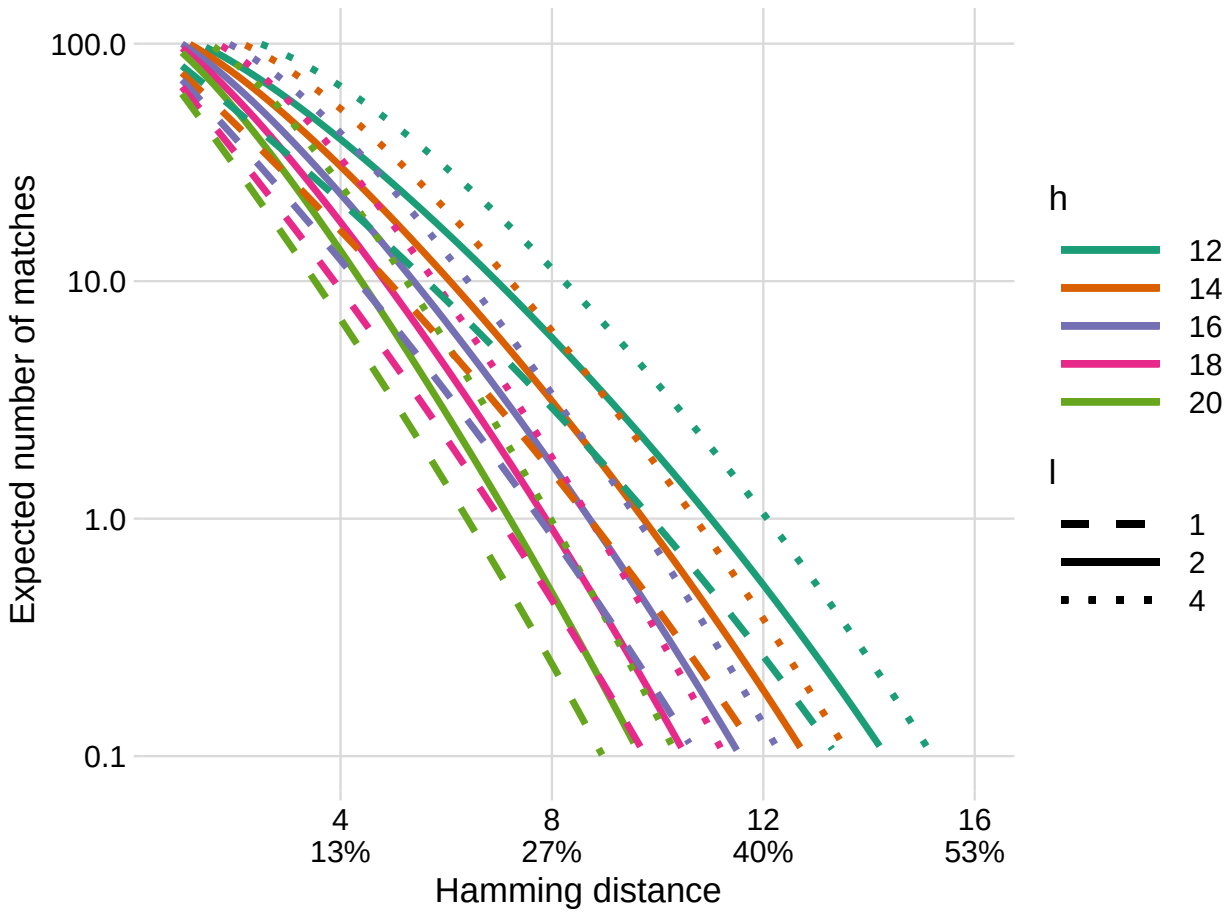


Figure S10: The expected number of 30-mers matched by the LSH approach for a short read of length $L{=}150$ as the normalized distance $^{d}/_{k}$ of the read to the closest match varies: $(L-k+1)\rho(d)$. Lines show different settings of l and h for an infinite b, i.e., all reference k-mers are stored in the library.