

1 RefSeq database growth influences the accuracy of k -mer-based 2 species identification

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9

10 ABSTRACT

11 Accurate species-level taxonomic classification and profiling of complex microbial communities
12 remains a challenge due to homologous regions shared among closely related species and a
13 sparse representation of non-human associated microbes in the database. Although the database
14 undoubtedly has a strong influence on the sensitivity of taxonomic classifiers and profilers, to
15 date, no study has carefully explored this topic on historical RefSeq releases and explored its
16 impact on accuracy. In this study, we examined the influence of the database, over time, on k -
17 mer based sequence classification and profiling. We present three major findings: (i) database
18 growth over time resulted in more classified reads, but fewer species-level classifications and
19 more species-level misclassifications; (ii) Bayesian re-estimation of abundance helped to recover
20 species-level classifications when the exact target strain was present; and (iii) Bayesian re-
21 estimation struggled when the database lacked the target strain, resulting in a notable decrease in
22 accuracy. In summary, our findings suggest that the growth of RefSeq over time has strongly
23 influenced the accuracy of k -mer based classification and profiling methods, resulting in

24 different classification results depending on the particular database used. These results suggest a
25 need for new algorithms specially adapted for large genome collections and better measures of
26 classification uncertainty.

27

28 Keywords: Taxonomic classification, Reference database, Metagenomics, Microbiome,
29 Comparative analysis

30

31 **INTRODUCTION**

32 Fundamental questions of a metagenomic survey are: *(i)* what microbes are present in each
33 sample, *(ii)* how abundant is each organism identified in a sample, *(iii)* what role might each
34 microbe play (i.e. what gene functions are present) and *(iv)* how do the previous observations
35 change across samples and time? Specifically, there have been numerous studies highlighting
36 the utility of metagenomic datasets for pathogen detection, disease indicators, and health ^{1,2}.
37 Addressing each of these fundamental questions is predicated on the ability to assign taxonomy
38 and gene function to unknown sequences.

39

40 Several new tools and approaches for taxonomic identification of DNA sequences have emerged
41 ³⁻⁵, in addition to community-driven ‘bake-offs’ and benchmarks ⁶. *k*-mer based classification
42 methods such as Kraken or CLARK ^{3,7} are notable for their exceptional speed and specificity, as
43 both are capable of analyzing hundreds of millions of short reads (ca. 100 base pairs) in a CPU
44 minute. These *k*-mer based algorithms use heuristics to identify unique, informative, *k*-length
45 subsequences (*k*-mers) within a database to help improve both speed and accuracy. A challenge
46 for *k*-mer based classification approaches is that closely related species and strains often contain

47 many identical sequences within their genomes. This challenge is typically addressed by
48 assigning the query sequence with the lowest common ancestor (LCA) of all species that share
49 the sequence. A comprehensive benchmarking survey indicated that Kraken offered the best F_1
50 score (a measure considering both precision and recall) among the k -mer based taxonomic
51 classifiers evaluated at the species level ⁸. Bracken, a Bayesian method that refines Kraken
52 results, is capable of estimating how much of each species is present among a set of ambiguous
53 species classifications by probabilistically re-distributing reads in a taxonomic tree ⁹. We thus
54 selected Kraken and Bracken as representative tools from the genre of k -mer based classification
55 methods. The focus on this study was not to examine a specific software tool, but rather to
56 decouple the performance of k -mer based methods from the underlying database.

57
58 Available k -mer based methods for taxonomic identification and microbiome profiling rely on
59 existing reference databases. While several investigations have examined the influence of
60 contamination in specific database releases, and identified idiosyncrasies specific to a release
61 ^{10,11}, no study has examined the specific influence of perhaps the most popular database from
62 which to build classification databases, the repository of sequenced and assembled microbes
63 (RefSeq), across all releases of the database. Additionally, metagenomic classification and
64 profiling tools are commonly compared to each other using simulated datasets on a fixed
65 database, with leave-one-out analysis, but never compared to each other across recent trajectories
66 in database growth. The aim of this study was to elucidate the influence of RefSeq database
67 growth over time on the performance of k -mer based taxonomic identification tools.

68

69 **RESULTS**

70 **RefSeq database growth**

71 Since its release in June 2003 bacterial RefSeq, on average, has doubled in size (giga base pairs,
72 Gbp) every 1.5 years (Fig. 1A), with the number of unique 31-mers in the database growing at a
73 similar rate (Fig. 1B). A more recent release, bacterial RefSeq version 84 (released 9/11/2017),
74 totaled over 700 Gbp of sequence data. The Simpson's index of diversity is a metric with values
75 between zero and one that reports the probability that two individuals randomly selected from a
76 sample will not belong to the same species. Samples with a high Simpson's index of diversity
77 (i.e. closer to one) may be considered more diverse than those with low values (i.e. closer to
78 zero). The diversity for each version of the bacterial RefSeq database increased until April 2013
79 where the Simpson's index of diversity for each subsequent bacterial RefSeq release has trended
80 downward (Fig. 1C). A slower growth is also seen in the number of new bacterial species in
81 each RefSeq version, indicating many of the same species are being sequenced repeatedly (Fig.
82 1D).

84 **Taxonomic classification over time with a simulated metagenome**

85 Kraken's own simulated validation set of ten known genomes was searched against nine versions
86 of bacterial RefSeq (1, 10, 20, 30, 40, 50, 60, 70, 80) and the MiniKraken database (4GB
87 version) (Fig. 2). The accuracy of each Kraken run depends on the RefSeq version used in the
88 search (Fig. 2; Table 1). Correct genus-level classifications increased as RefSeq grew, but
89 correct species-level classifications peaked at version 30 and tended to decline thereafter (Fig. 2).
90 The decrease in correct species classifications is due to more closely-related genomes appearing
91 over time in RefSeq, making it difficult for the classifier to distinguish them and forcing a move
92 up to the genus level. Overall, misclassified species-level calls were consistently rare, as reads

93 were misclassified at the species level an average of 7% of the time (Table 1; Fig. 2). The
94 fraction of reads classified at any taxonomic level, regardless of accuracy, increases as RefSeq
95 grows over time (Fig. 3). However, the fraction of species-level assignments (again, regardless
96 of accuracy) peaks at RefSeq version 30 and begins to decline thereafter, while the fraction of
97 genus-level classifications begins to increase.

98
99 Bracken was used to re-estimate the abundances of classifications made by Kraken when
100 searching the simulated reads against eight bacterial RefSeq database versions (1, 10, 20, 30, 40,
101 50, 60, 70). Bracken first derives probabilities that describe how much sequence from each
102 genome is identical to other genomes in the database. This step requires searching a Kraken
103 database against itself with Kraken, which could not be performed for the MiniKraken DB (as
104 there is no FASTA file for this database) or bacterial RefSeq version 80 (as it would require
105 extensive computation for a database that size). Bracken was able to re-estimate species
106 abundances for 95% of the input data using RefSeq version 70, while Kraken only classified
107 51% of reads at the species level. Because Bracken may probabilistically distribute a single
108 read's classification across multiple taxonomy nodes, its performance must be measured in terms
109 of the predicted abundances. Bracken typically included the correct species in its re-estimation,
110 but sometimes included incorrect species in the abundance estimation (on average 15% of reads
111 were associated with a genome outside of the ten knowns).

112

113 **Taxonomic classification of difficult to classify genomes over time**

114 The challenging nature of classifying sequences belonging to the *Bacillus cereus* sensu lato
115 group has been previously documented^{12,13}. The *B. anthracis* species within this group is a well-

116 defined monophyletic subclade of the larger *B. cereus* group, and the base of the *B. anthracis*
117 clade is commonly denoted by a single nonsense mutation in the *plcR* gene¹⁴ which is conserved
118 in all known *B. anthracis* genomes and has been shown to confer a regulatory mutation essential
119 for maintaining the pXO1 and pXO2 plasmids that carry the virulence factors characteristic of
120 anthrax¹⁵. However, not all *B. anthracis* cause disease in humans, such as *B. anthracis* Sterne
121 (missing the pXO2 plasmid) and some *B. cereus* strains do cause anthrax-like disease¹⁶,
122 complicating a precise species definition. Thus, it is not a surprise that accurate species-level
123 classification within this group has proven challenging for *k*-mer based methods, especially those
124 methods not based on phylogenetic evidence. To demonstrate how difficult sequences from this
125 group have been to classify over time, simulated reads were created for two *Bacillus cereus*
126 strains. The first, *B. cereus* VD118, is a strain available in RefSeq version 60 and beyond, and
127 the second, *B. cereus* ISSFR-23F¹⁷, was recently isolated from the International Space Station
128 and is not present in any of the RefSeq releases tested. It is phylogenetically close to *B.*
129 *anthracis*, but lacks the phylogenetic and species characteristics of *B. anthracis*. Again, as
130 bacterial RefSeq grows over time, the number of genus-level classifications made by Kraken
131 increases (Fig. 4). While the number of genus-level calls made by Kraken increases over time
132 the number of unclassified and misclassified species calls decreases (most commonly *B.*
133 *anthracis*, *B. thuringensis*, and *B. weihenstephanensis*).

134
135 Bracken made species-level predictions for all reads no matter which version of bacterial RefSeq
136 was used (Fig. 4). However, the increased rate of species-level predictions came at the cost of
137 accuracy, as Bracken correctly identified *B. cereus* VD118 and *B. cereus* ISSFR-23F an average
138 of 72% and 29% of the time, respectively, across RefSeq versions 1 through 70. The fraction of

139 reads assigned to each *Bacillus* species varied substantially from each database tested. The range
140 of *Bacillus* species predictions for *B. cereus* VD118 were: *B. cereus* 81% (max=100%,
141 min=18%), *B. anthracis* 48% (max=48%, min=0%), *B. thuringiensis* 23% (max=23%, min=0%),
142 and *B. weihenstephanensis* 76% (max=76%, min=0%). While the range of *Bacillus* species
143 predictions for *B. cereus* ISSFR-23F were: *B. cereus* 45% (max=50%, min=5%), *B. anthracis*
144 90% (max=95%, min=5%), and *B. thuringiensis* 54% (max=54%, min=0%).

145

146 **CPU/Memory performance over time**

147 Historical bacterial RefSeq versions were recreated and used to build Kraken databases with
148 default settings. While most databases were constructed with ease and in less than a day, version
149 70 required 500 GB of RAM and 2 days (single compute node using on 64 cores), while version
150 80 required ca. 2.5 TB of RAM and ca. 11 days (single compute node using on 64 cores). Given
151 this trend, future releases will likely require over 4 TB of RAM and weeks of computation to
152 build, putting into question the feasibility of building and profiling *k*-mer databases on future
153 RefSeq versions. Recent studies¹⁸ have suggested alternative approaches for database
154 construction that would help to circumvent future computational bottlenecks.

155

156 **DISCUSSION**

157 The results of our study support three conclusions: (i) the RefSeq bacterial database composition
158 and diversity is dynamic, varying from release to release; (ii) the database composition strongly
159 influences the performance of *k*-mer based taxonomic identification methods, and (iii) Bayesian
160 based methods can help mitigate some of the effect, but struggle with novel genomes that have
161 close relatives in the database.

162

163 *Database influences on k-mer based taxonomic classification*

164 Using Bracken, the majority of *Bacillus cereus* ISSFR-23F simulated reads were not correctly
165 assigned to *B. cereus* but were more frequently mis-assigned as *Bacillus anthracis* or *Bacillus*
166 *thuringiensis* (Fig. 4B). This, in part, is not surprising as two of the three species in this group, *B.*
167 *cereus* and *B. thuringiensis*, have no clear phylogenetically defined boundary, though *B.*
168 *anthracis* is phylogenetically distinct from *B. cereus* and *B. thuringiensis*. Furthermore, any two
169 genomes within the *Bacillus cereus* sensu lato group are likely to be over 98% identical⁹. Given
170 that *k*-mer based methods are not phylogenetically-grounded, but rather based on sequence
171 composition, they are susceptible to misidentification in clades where the taxonomy is in partial
172 conflict with phylogeny, such as the *Bacillus cereus* sensu lato group. One clear example of
173 misidentification within this group was the false identification Anthrax in public transit systems
174 ^{19,20}.

175

176 Another observation worth highlighting is that the fraction of simulated reads classified as one of
177 the three *B. cereus* sensu lato species varied across database versions (Fig. 4), with the exception
178 of *B. cereus* VD118, which was present in RefSeq releases 60 and 70 (Fig. 4A). The variation in
179 species classifications across database versions indicates that even when using the same tools to
180 analyze the same dataset, the conclusions derived from this analysis can vary substantially
181 depending on which version of a database you are searching against, especially for genomes
182 belonging to difficult to classify species (i.e. require phylogenetic-based approaches).

183

184 *Imperfect data*

185 The genomic data deluge has helped to expand public repositories with a broader and deeper
186 view of the tree of life, but has also brought with it contamination and misclassification.
187 Contamination in public databases is well-documented²¹ and represents an additional
188 confounding factor for *k*-mer based methods. While several custom tools have been built to deal
189 with imperfect data²², there is a need for database ‘cleaning’ tools that can preprocess a database
190 and evaluate it for both contamination (genome assemblies that contain a mixture of species) and
191 misclassified species and strains (genomes that are assigned a taxonomic ID that is inconsistent
192 with its similarity to other genomes in the database). The misclassification issue often is in the
193 eye of the beholder; species have been named based on morphology, ecological niche, toxin
194 presence/absence, isolation location, 16S phylogenetic placement, and average nucleotide
195 identity across the genome. This, coupled with an often ambiguous species concept in microbial
196 genomes due to horizontal gene transfer and mobile elements²³, brings into question the reliance
197 on the current taxonomic structure for assigning names to microbes sequenced in metagenomic
198 samples. A more robust approach would be for the classification databases to derive their own
199 hierarchical structure directly from the data, rather than taxonomy, and then map back the
200 internally derived hierarchy to widely-used taxonomic names.

201

202 **CONCLUSION**

203 Our findings demonstrate that changes in RefSeq over time have influenced the accuracy of *k*-
204 mer based classification and profiling methods. Bayesian re-estimation approaches are helpful
205 for species or strain level prediction but can result in false positives and are computationally
206 prohibitive with larger databases. Despite recent progress in *k*-mer based methods for
207 metagenome profiling and classification these tools should likely be used as step one in a multi-

208 step process, which also includes read mapping, assembly, feature prediction, and annotation.
209 Additionally, priority should be given to the breadth, not depth, of species added to reference
210 databases over time.

211

212 **METHODS**

213 **Acquisition of bacterial RefSeq databases versions 1 through 80**

214 FASTA files of previous versions of bacterial RefSeq are not publically available for download.
215 Therefore, sequences from previous versions of bacterial RefSeq were acquired using custom
216 scripts (https://github.com/dnasko/refseq_rollback). Briefly, the process involved downloading
217 the current bacterial RefSeq release (ver. 84 as of the date of the analysis) FASTA files
218 (<ftp.ncbi.nlm.nih.gov/refseq/release/bacteria>) and concatenating them into one file. Then, the
219 catalog file associated with the desired version is downloaded
220 (<ftp.ncbi.nlm.nih.gov/refseq/release/release-catalog/archive>), which contains the identifiers for
221 sequences present in that version of bacterial RefSeq. Sequence identifiers in that version's
222 catalog file are pulled from the current RefSeq FASTA file and written to a new file. Using the
223 `refseq_rollback.pl` script any version of bacterial RefSeq can be created. For this study only
224 versions 1, 10, 20, 30, 40, 50, 60, 70, and 80 were recreated.

225

226 **Taxonomic classification on simulated datasets**

227 Two simulated read datasets were used to test Kraken and Bracken performance with different
228 versions of the bacterial RefSeq database. The first simulated dataset was downloaded from the
229 Kraken website (ccb.jhu.edu/software/kraken) and was previously used in the Kraken manuscript
230 as a validation set³. Briefly, this simulated dataset was composed of 10 known bacterial species:

231 *Aeromonas hydrophila* SSU, *Bacillus cereus* VD118, *Bacteroides fragilis* HMW 615,
232 *Mycobacterium abscessus* 6G-0125-R, *Pelosinus fermentans* A11, *Rhodobacter sphaeroides*
233 2.4.1, *Staphylococcus aureus* M0927, *Streptococcus pneumoniae* TIGR4, *Vibrio cholerae*
234 CP1032(5), and *Xanthomonas axonopodis* pv. Manihotis UA323. Each genome had 1,000
235 single-end reads (101 bp in size) for a total of 10,000 reads. We selected this dataset as it has
236 been widely used as a benchmark for other *k*-mer based classification methods^{3,7} and represents
237 a breadth of species. This simulated read dataset was classified against each of the recreated
238 bacterial RefSeq databases using Kraken (ver 1.0) with default settings.

239
240 To test the ability to classify reads from genomes not in the bacterial RefSeq database 10,000
241 simulated single-end Illumina reads (101 bp) were created using Grinder²⁴ with default settings
242 from: (i) a *Bacillus cereus* genome, *B. cereus* VD118, not present in RefSeq until version 60 and
243 beyond; and (ii) a novel *B. cereus* genome, *B. cereus* ISSFR-23F¹⁷, never present in any of the
244 RefSeq versions tested. We decided to use these genomes as they are members of the *B. cereus*
245 sensu lato group, containing a collection of species that are known to be challenging for *k*-mer
246 methods to distinguish between^{19,20}. These datasets were classified with Kraken (ver. 1.0) and
247 Bracken (ver. 1.0.0)⁹ both with default settings (Bracken “read-length” set to 101).

248

249 **Running Bracken on Kraken output**

250 Bracken (ver. 1.0.0) was run on the output of each Kraken search (except for release 80 and
251 KrakenMiniDB). Default parameters were used except for “read-length”, which was set to 101.

252

253 **Bacterial RefSeq diversity metric calculations**

254 Diversity metrics were calculated for every version of bacterial RefSeq (1-84) by parsing the
255 catalog files for each version. An operational taxonomic unit (OTU) table was constructed using
256 the NCBI taxonomy identifiers as taxonomic units (see `create_otu_table.pl` in the `refseq_rollback`
257 repository). The OTU table was imported to QIIME (ver. MacQIIME 1.9.1-20150604)²⁵.
258 Diversity metrics (Simpson, Shannon, Richness) were calculated using the “`alpha_diversity.py`”
259 script and plotted using the R base package.

260

261 **ABBREVIATIONS**

262 OTU: Operational taxonomic unit; LCA: Lowest common ancestor

263 **DECLARATIONS**

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277 expressed or implied, of the ODNI, IARPA, ARO, or the US Government.

278

279 **Availability of Data and Materials**

280 Scripts used in this analysis are available on GitHub
281 (github.com/dnasko/refseq_rollback). Datasets and genomes used in this analysis are
282 available online and referenced in the text.

283

284 **Authors' contributions**

285 T.J.T. and D.J.N. designed the experiments. D.J.N. wrote the analysis scripts. D.J.N.,
286 S.K, and T.J.T. performed the experiments. D.J.N., S.K., A.M.P. and T.J.T. wrote the
287 paper.

288

289 **Ethics approval and consent to participate**

290 NA

291 **Consent for publication**

292 NA

293 **Competing interests**

294 NA

295 **Additional files**

296

297 **REFERENCES**

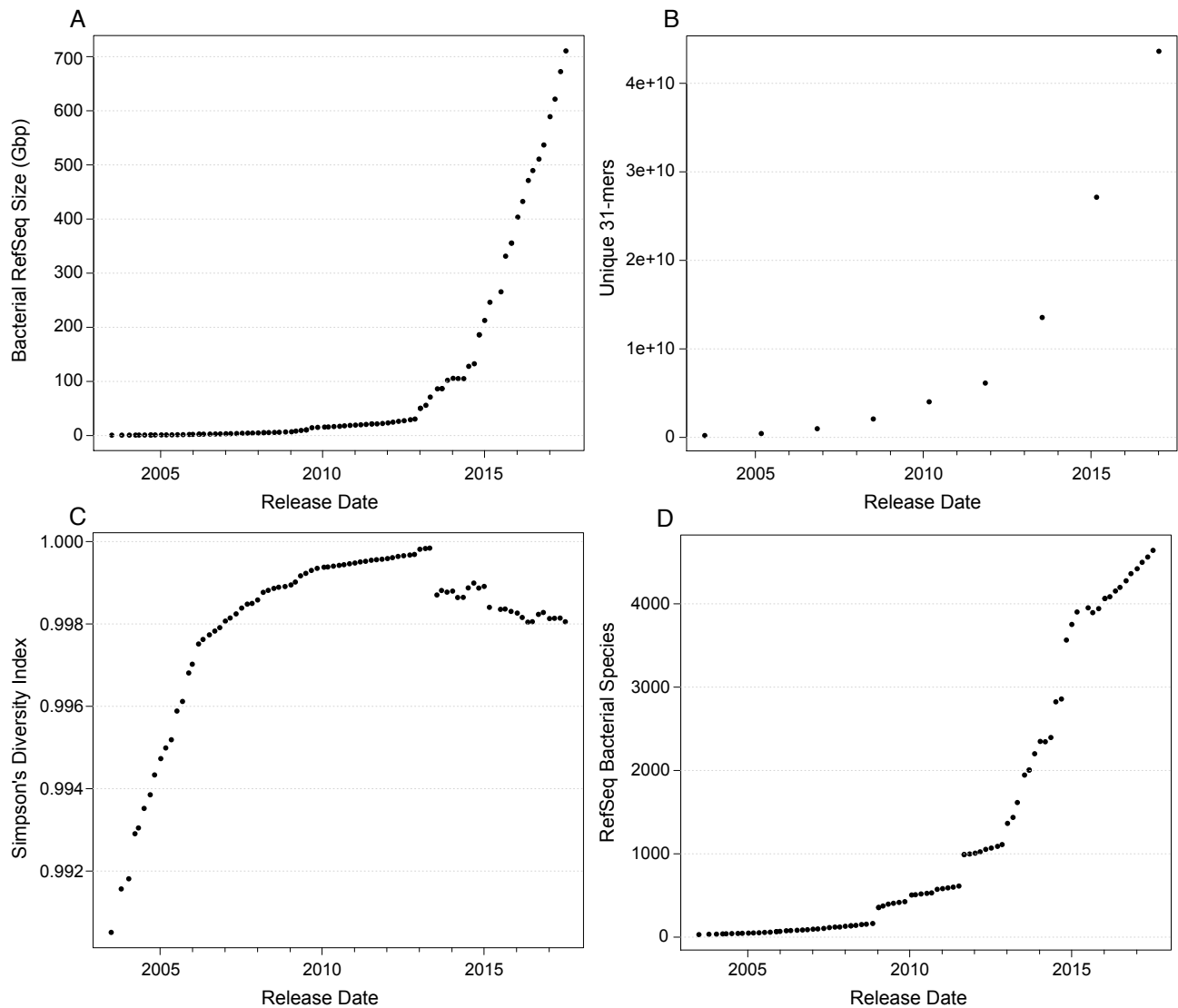
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- 358
- 359

360 **Table 1. Fractions of unclassified (Unclass.), correctly classified (Correct), and misclassified**
361 **(Misclass.) simulated reads from ten genomes using Kraken against different versions of**
362 **bacterial RefSeq.**

Release	Date	Genus			Species	
		Unclass.	Correct	Misclass.	Correct	Misclass.
1	2003-06-30	0.62	0.38	0.00	0.29	0.08
10	2005-03-06	0.53	0.46	0.01	0.38	0.07
20	2006-11-05	0.49	0.49	0.01	0.40	0.08
30	2008-07-07	0.25	0.74	0.00	0.60	0.07
40	2010-05-07	0.22	0.77	0.00	0.54	0.08
50	2011-11-08	0.21	0.78	0.01	0.52	0.07
60	2013-07-19	0.03	0.96	0.00	0.57	0.09
70	2016-03-03	0.03	0.95	0.01	0.42	0.09
80	2017-01-09	0.03	0.94	0.01	0.28	0.08

363



364

365 **Figure 1: Simpson diversity index of bacterial RefSeq has decreased every release since**

366 **April 2013.** (A) The number of base pairs in bacterial RefSeq continues to grow exponentially,

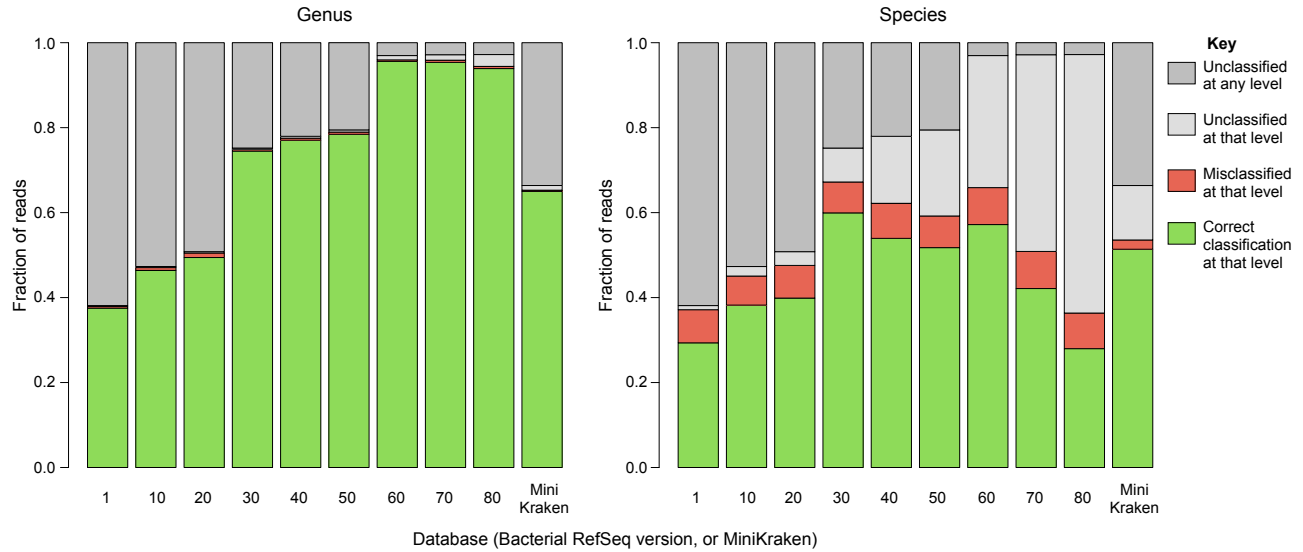
367 but (B) the number of unique 31-mers and (D) the number of bacterial species added increases

368 slower. (C) The Simpson's diversity index grew every release up to April 2013 where it has

369 declined every release since.

370

371



372

373 **Figure 2: The fraction of correct species classifications (right) decreases in later RefSeq**
374 **database versions because they are only being classified at the genus level (left). Kraken**

375 classification results of simulated reads from known genomes against nine versions the bacterial
376 RefSeq database and the MiniKraken database. Misclassifications at the genus and species
377 levels remain consistently low across database versions.

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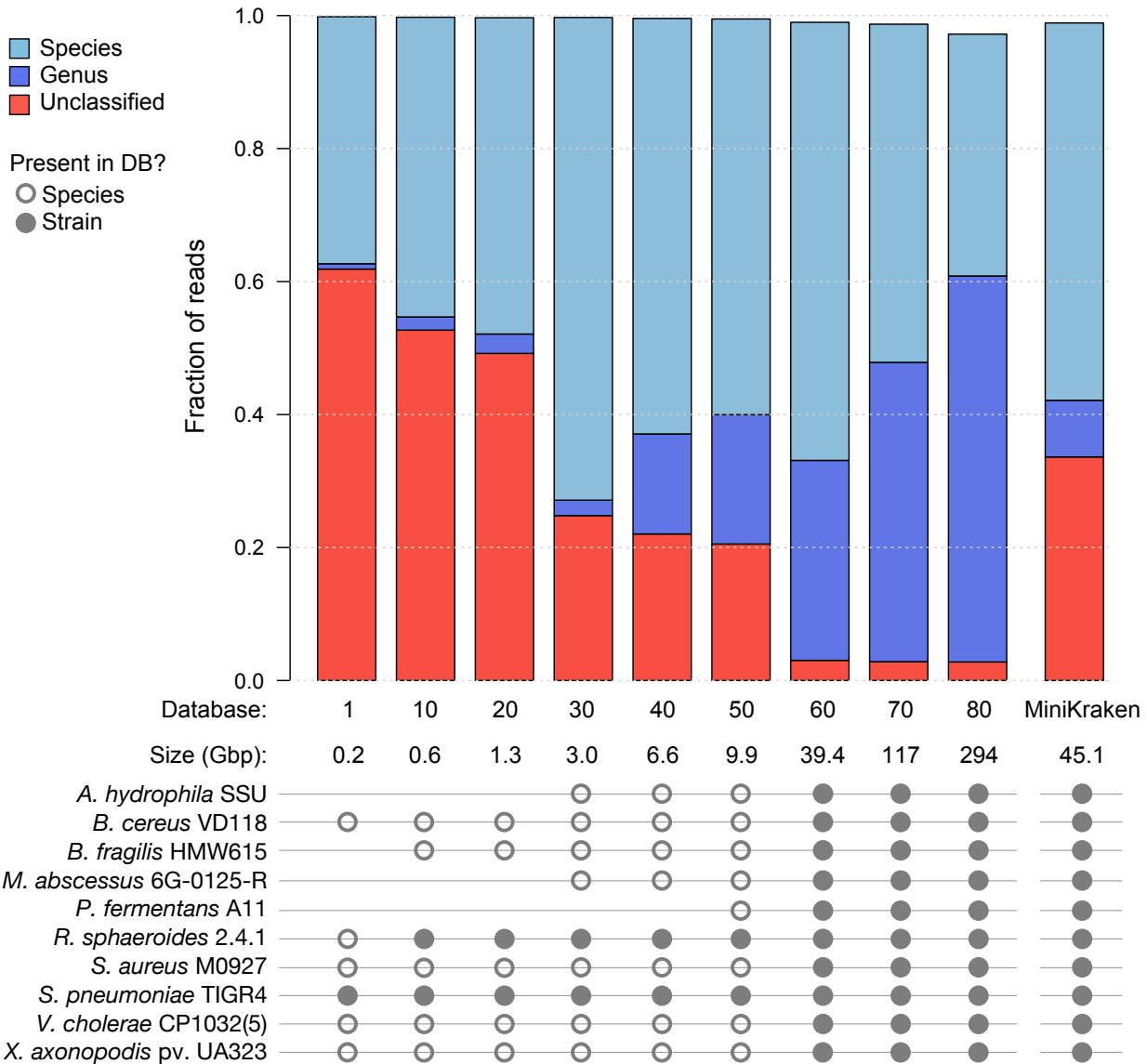
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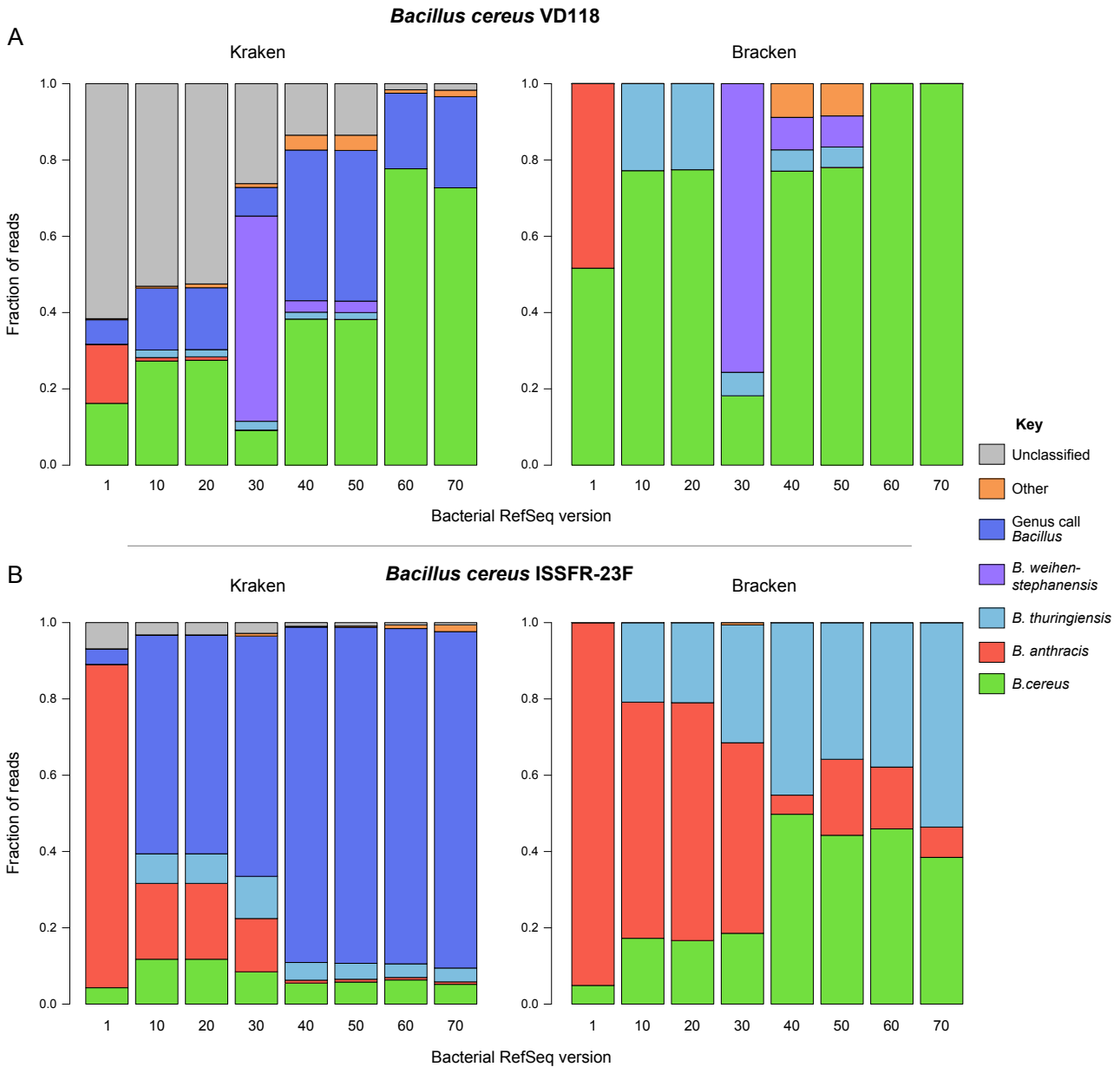


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389 **Figure 3: Species-level classifications decrease, and genus-level classifications increase as**
 390 **bacterial RefSeq grows.** Fraction of simulated reads classified at different taxonomic levels,
 391 regardless of accuracy, using Kraken against ten databases. The circles below indicate when
 392 each genome's species/strain is in a database. Although the MiniKraken database contains all 10
 393 genomes it yields results comparable to bacterial RefSeq version 40.

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397 **Figure 4: The fraction of simulated reads classified among *Bacillus* species varied**
 398 **considerably depending on which RefSeq version was used, demonstrating the influence of**
 399 **the database on a *k*-mer based taxonomic classification. (A) Classifying simulated *B. cereus***
 400 **VD118 reads with Kraken (left) and Bracken (right) against different version of RefSeq.**
 401 **Species-level classifications varied, and the fraction of unclassified reads decreased with Kraken,**

402 as the database grew. Once *B. cereus* VD118 appeared in the database (ver. 60) Bracken
403 correctly classified every read. **(B)** Species-level classifications decrease with Kraken as RefSeq
404 grows using simulated reads from an environmental *Bacillus cereus* not in RefSeq. Fraction of
405 simulated *B. cereus* ISSFR-23F reads classified using Kraken ver. 1.0 (left) and Bracken ver.
406 1.0.0 (right) against different versions of bacterial RefSeq. Bracken classification pushed all
407 reads to a species-level call, though these classifications were often for other *Bacillus* species.
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