

1   **MetaCerberus: distributed highly parallelized scalable HMM-based**  
2   **implementation for robust functional annotation across the tree of**  
3   **life**

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25 **Abstract**

26 **Summary:** MetaCerberus is an exclusive HMM/HMMER-based tool that is massively  
27 parallel, on low memory, and provides rapid scalable annotation for functional gene  
28 inference across genomes to metacommunities. It provides robust enumeration of  
29 functional genes and pathways across many current public databases including KEGG  
30 (KO), COGs, CAZy, FOAM, and viral specific databases (i.e., VOGs and PHROGs). In a  
31 direct comparison, MetaCerberus was twice as fast as EggNOG-Mapper, and produced  
32 better annotation of viruses, phages, and archaeal viruses than DRAM, PROKKA, or  
33 InterProScan. MetaCerberus annotates more KOs across domains when compared to  
34 DRAM, with a 186x smaller database and a third less memory. MetaCerberus is fully  
35 integrated with differential statistical tools (i.e., DESeq2 and edgeR), pathway  
36 enrichment (GAGE R), and Pathview R for quantitative elucidation of metabolic  
37 pathways. MetaCerberus implements the key to unlocking the biosphere across the tree  
38 of life at scale.

39 **Availability and implementation:** MetaCerberus is written in Python and distributed under  
40 a BSD-3 license. The source code of MetaCerberus is freely available at  
41 <https://github.com/raw-lab/metacerberus>. Written in python 3 for both Linux and Mac OS  
42 X. MetaCerberus can also be easily installed using mamba create -n metacerberus -c  
43 bioconda -c conda-forge metacerberus

44  
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46 **Supplementary information:** Supplementary data are available online.

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48 **Introduction**

49 Annotation is a fundamental step in functional gene inference, which is required  
50 by many disciplines in biology. Massively parallel sequencing (MPS) has reached the  
51 terabyte scale with Illumina NovaSeq X producing 16 Tb per run and Oxford nanopore  
52 promethION 7 Tb per run (1-2). Due to this increase in MPS, the number of reference  
53 microbial genomes and metagenomes has increased by orders of magnitude. Genome  
54 Taxonomy Database (GTDB) now includes 402,709 (08-RS214, April 28<sup>th</sup>, 2023)  
55 genomes, and the Short Read Archive (SRA) has >4.5 million listed biosample  
56 metagenomes (3-4). Cellular metagenome-assembled genomes (MAGs) and their viral  
57 counterpart vMAGs (viral MAGs) have also rapidly populated public databases through  
58 reconstruction from shotgun metagenomics (5-7). Functional gene annotation is  
59 required for metabolic reconstruction, functional and structural gene differential analysis,  
60 inference of pathway regulation, presence/absence of toxin genes (e.g., botulinum toxin  
61 A), novel gene cluster discovery (e.g., antibiotic discovery), and viral detection. Due to  
62 this Terabyte scale, the annotation step will be the most prolonged, requiring more CPU  
63 time, memory, and resources to finish before obtaining biological insight. Reference  
64 databases have also been nearing the Terabyte scale, taking days to download and  
65 format, requiring massive allocations of disk space. Thus scalable, highly parallel, low  
66 memory, and rapid annotation tools are critical to the future of 'omics analysis.

67 Functional annotation requires two main steps: 1) gene calling followed by 2)  
68 gene assignment via external reference databases. Multiple approaches have been  
69 applied for gene calling and gene assignment, including homology and ontology-based  
70 methods. Gene calling finds protein-coding open reading frames (pORFs) alongside

71 ribosomal RNAs, transfer RNAs, and other RNAs. Various tools exist for pORF calling,  
72 including Prodigal, FragGeneScanRs, GetOrf, and GeneMark (8-11). Gene assignment  
73 of pORFs to external databases often uses homology-based tools such as BLAST (12),  
74 MMseq2 (13), and/or DIAMOND (14) against databases such as RefSeq (NCBI  
75 Reference Sequence Database) (15), UniProt (Universal Protein Resource) (16), or  
76 KEGG (Kyoto Encyclopedia of Genes and Genomes) (17). Common tools include  
77 PROKKA, DRAM (Distilled and Refined Annotation of Metabolism), InterProScan  
78 (INTEgrative PROtein signature database), EggNOG-Mapper (evolutionary genealogy  
79 of genes: Non-supervised Orthologous Groups), and MicrobeAnnotator (18-22).  
80 Ontology-based approaches are generally superior to homology-based methods (21).  
81 EggNOG-Mapper and InterProScan utilize homology-based (i.e., Diamond and BLAST)  
82 and Hidden Markov Models (HMMs) based ontology approaches via HMMER (23) using  
83 either KEGG (17), eggNOG (24), InterPro (25), or Pfam (protein family) databases (26).  
84 HMMs provide greater sensitivity to elucidate and discover relationships between query  
85 and database based on ontology and are protein domain-centric (21, 27).

86 Viruses and the candidate phyla radiation (CPR) have remained challenging to  
87 functionally annotate due to the divergent nature of their proteins (28-29). DRAM has a  
88 specific version (i.e., DRAM-v) to annotate viruses, including the detection of viral  
89 auxiliary metabolic genes (vAMGs) (19). MicrobeAnnotator and DRAM have attempted  
90 to close the gap in CPR functional annotation. While no specific annotation tool or gene  
91 database exists for CPR, they are found amongst GTDB and other public repositories  
92 (30). Various databases such as VOGs (Virus Orthologous Groups), pVOGs  
93 (Prokaryotic Virus Orthologous Groups), IMG/VR (Integrated Microbial Genome/Virus),

94 INPHARED (INfrastructure for a PHAge REference Database), and PHROGs  
95 (Prokaryotic Virus Remote Homologous Groups database) have been introduced to  
96 improve annotation viruses from isolates and vMAGs (31-35). Still, CPR and viruses  
97 remain a significant challenge for functional annotation.

98 Many tools are available for functional annotation from genomes to  
99 metagenomes; however, gaps remain between: 1) resource utilization (e.g., memory  
100 use), 2) large database size, and 3) parallel processing, and simultaneously providing  
101 robust rapid annotation at scale. Further development of tools for CPR and viral  
102 functional annotation are a general community need. We present MetaCerberus, an  
103 ontology-based HMM tool that provides scalable, highly parallel, low memory usage,  
104 and rapid annotation for genomes to metacommunities across the tree of life.

105 **Implementation**

106 *Framework and coding base*

107 MetaCerberus is written entirely in Python (version 3) as a wrapper for various  
108 other tools described below. Similar to our other software MerCat2 for massively parallel  
109 processing (MPP), it utilizes a byte chunking algorithm 1 ('Chunker') to split files for  
110 MPP for further utilization in RAY, a massive open-source parallel computing framework  
111 to scale Python applications and workflows (36). Using RAY's scalable parallelization  
112 within MetaCerberus allows utilization across multiple nodes with ease. To avoid large  
113 RAM consumption, we implemented the greedy algorithm for tab-separated merging  
114 and incremental PCA plot limiter from MerCat2 (36). MetaCerberus utilizes  
115 HMM/HMMER exclusively without homology-based tools (e.g., BLAST)  
116

117 *Databases for MetaCerberus*

118        MetaCerberus enables functional gene assignment across multiple databases,  
119        including: 1) KOfams (KEGG protein families) to obtain KEGG KOs (KEGG Ontology)  
120        (version 11-Jul-2023, <https://www.genome.jp/ftp/db/kofam/>), 2) FOAM (Functional Ontology  
121        Assignments for Metagenomes), 3) COG (Clusters of Orthologous Genes) (version  
122        2020, <https://ftp.ncbi.nih.gov/pub/COG/COG2020/data/>), and 4) dbCAN (DataBase for  
123        automated Carbohydrate-active enzyme ANnotation) for CAZy (Carbohydrate-Active  
124        enZYmes Database) (version 11, <https://bcb.unl.edu/dbCAN2/download/>) (**37-41**). For viral  
125        annotation, MetaCerberus enlists VOG (version 219, <https://vogdb.org/download>), pVOG  
126        (version Sep2016, <https://ftp.ncbi.nlm.nih.gov/pub/kristensen/pVOGs/downloads.html#>), and  
127        PHROG (version 4, <https://phrogs.lmge.uca.fr/>) databases. FOAM ontology is obtained  
128        from KOfam KOs, and then computed via a reference table to avoid redundancy.  
129        Similarly, the dbCAN database is used to obtain CAZy ontology via a reference table.  
130        COGs and PHROGs are currently not formatted as HMMs within their public  
131        repositories. We converted them into protein family-specific HMMs (e.g., COG1 ->  
132        COG1.hmm) using MAFFT (version 7.273-woe) via local alignments with maximum  
133        iterations of 1000 (**42**).        We compared databases of six other tools to MetaCerberus,  
134        including DRAM, PROKKA, InterProScan, MicrobeAnnotator, and EggNOG-Mapper  
135        (**Table 1**). Currently, only MetaCerberus provides functional annotation and support to  
136        FOAM, pVOG, and PHROG databases (**Table 1**). EggNOG-Mapper and MetaCerberus  
137        are the only tools we compared that supports the COG database (**Table 1**). All tools  
138        compared in this study obtain the enzyme commission numbers (EC) numbers (**Table  
139        1**).

140 *Modes for running MetaCerberus*

141        MetaCerberus has three basic modes: 1) quality control (QC) for raw reads, 2)  
142        formatting/gene prediction, and 3) annotation (**Fig 1**). MetaCerberus can use three  
143        different input files: 1) raw read data from any sequencing platform (Illumina, PacBio, or  
144        Oxford Nanopore), 2) assembled contigs, as MAGs, vMAGs, isolate genomes, or a  
145        collection of contigs, 3) amino acid fasta (.faa), previously called pORFs (**Fig 1**). We  
146        offer customization, including running databases all together, individually or specifying  
147        select databases. For example, if a user wants to run a prokaryotic or eukaryotic-  
148        specific KOfam, or an individual database alone such as dbCAN, both are easily  
149        customized within MetaCerberus. In future versions, we will provide viral and phage-  
150        specific KO modules to run individually. In QC mode, raw reads are quality controlled  
151        via fastqc (version v0.12.1) prior and post trim (**43**). Raw reads are then trimmed via  
152        data type; if the data is Illumina or PacBio, fastp (version 0.23.4) is called, otherwise it  
153        assumes the data is Oxford nanopore then Porechop (version v0.2.4) is utilized (**43-45**,  
154        **Fig 1**). Post quality-control trimmed reads are converted to fasta without quality (**Fig 1**).  
155        If Illumina reads are utilized, an optional bbmap (version 39.01) step to remove the  
156        phiX174 genome is available. Phage phiX174 is a common contaminant within the  
157        Illumina platform as it is their library spike-in control (**46-47**). We highly recommend this  
158        removal if viral analysis is conducted, as it would provide false positives to ssDNA  
159        microviruses within the sample.

160        In the formatting and gene prediction mode, contigs and genomes are checked  
161        for N repeats. These N repeats are removed by default. We impute contig/genome  
162        statistics (e.g., N50, N90, max contig) via our custom module Metaome Stats. Contigs

163 are converted to pORFs via Prodigal or FragGeneScanRs as specified by user  
164 preference (48, Fig 1). Scaffold annotation is not recommended due to N's providing  
165 ambiguous annotation. Both callers can be used via our --super option, and we  
166 recommend using FragGeneScanRs for samples rich in eukaryotes as it performed  
167 better in our hands than Prodigal (unpublished data). HMMER searches against the  
168 above databases via user specified bitscore and e-values or our minimum defaults (i.e.,  
169 bitscore = 25, e-value =  $1 \times 10^{-9}$ ).

170 There are six general rules followed by MetaCerberus for functional annotation.

171 Rule 1 is the *score pre-filtering module* for pORFs thresholds: each pORF match to an  
172 HMM is recorded by default or user-selected e-value/bit scores per database  
173 independently, across all databases, or per user specification of the selected database.  
174 Rule 2 is imputed for *non-overlapping dual domain module* pORF threshold: if two HMM  
175 hits are non-overlapping from the same database, both are counted as long as they are  
176 the within the default or user selected e-value/bit scores. Rule 3 is computed as the  
177 *winner take all module* for overlapping pORFs: if two HMM hits are overlapping ( $>10$   
178 amino acids) from the same database the lowest resulting e-value and highest bit score  
179 wins. Rule 4 is *similar match independent accession module* for a single pORF: if both  
180 hits within the same database have equal values for both e-value and bit score but are  
181 different accessions from the same database (e.g., KO1 and KO3) then both are  
182 reported. Rule 5 is the *whole count incomplete exclusion module* filter only allows  
183 whole discrete integer counting. Rule 6 the *dual independent overlapping domain*  
184 *module* for convergent binary domain pORFs. If two domains within a pORF are  
185 overlapping  $<10$  amino acids (e.g., COG1 and COG4) then both domains are counts and

186 reported due to the dual domain issue within a single pORF. If a function hits multiple  
187 pathways within an accession, both are counted, in pathway roll-up, as many proteins  
188 function in multiple pathways.

189

190 *Statistics and visualization*

191 MetaCerberus, as previously mentioned, provides genome and contig statistics  
192 via MetaOme stats; it also offers seamless integration into automatic differential  
193 statistics, visualizations, pathway enrichment, and pathway integration viewing. DESeq2  
194 and edgeR negative binomial distribution differential statistic tools are available to users  
195 by selection (default is DESeq2) (49-50). The outputs from DESeq2, edgeR, or both are  
196 automatically enriched for pathway analysis in GAGE (Generally Applicable Gene-set  
197 Enrichment for Pathway Analysis) R (51). GAGE outputs are loaded into Pathview R to  
198 visualize differential pathways across user-specified experiments (52). These outputs  
199 include differential KEGG heatmaps from Pathview, volcano plots, and gene level  
200 heatmaps (**Fig S1**).

201 A sample dashboard visualization is provided for all data input types (e.g., reads,  
202 contigs and/or genomes) with a number of pORF called, MetaOme stats (i.e., genome  
203 statistics, N50, N90, etc., for genomes/contigs only), PCA with sample sets of >3, and  
204 the number of annotated hits for all databases or user select specifications (**Fig S1**).

205

206 *Across tool comparisons*

207 Tools compared across MetaCerberus (version 1.1) include DRAM (version  
208 1.4.6), InterProScan (version 5.60-92.0), EggNog-Mapper (version 2.1.8),

209 MicrobeAnnotator (version 2.0.5), and PROKKA (version 1.1). All comparisons were  
210 completed on a Dual 8-Core Intel Xeon E5-2667 CPU @ 3.2GHz (16 cores) using  
211 128GB RAM. MPP testing of MetaCerberus was completed on five nodes of a Dual 18-  
212 Core Intel Xeon Gold 6154 CPU @ 3.00GHz (36 cores/node). All genomes used in our  
213 study are available at <https://osf.io/3uz2j/>. For further testing of MetaCerberus, we used  
214 five distinct genospecies, *Rhizobium leguminosarum*, against five distinct  
215 *Exiguobacterium* spp. available at  
216 [https://github.com/raw-lab/MetaCerberus/tree/main/data/rhizobium\\_test](https://github.com/raw-lab/MetaCerberus/tree/main/data/rhizobium_test) (**Table S1**).  
217 Viruses from permafrost that were used in the DRAM paper  
218 (<https://www.ncbi.nlm.nih.gov/nuccore/QGNH00000000>) were compared directly to  
219 MetaCerberus and DRAM (**19**).  
220

221 *Data availability*  
222 Sequence files, genome files, and supplemental data are available at  
223 <https://osf.io/3uz2j/>. Databases are also freely available at <https://osf.io/3uz2j/>. All code is  
224 available at [www.github.com/raw-lab/metacerberus](https://www.github.com/raw-lab/metacerberus).

225  
226 *Contributing to MetaCerberus and Fungene*  
227 MetaCerberus is a community resource as is recently acquired FunGene  
228 (<http://fungene.cme.msu.edu/>). We welcome contributions of other experts expanding  
229 annotation of all domains of life (viruses, phages, bacteria, archaea, eukaryotes).  
230 Please send us an issue on our MetaCerberus GitHub.  
231 ([www.github.com/raw-lab/metacerberus/issue](https://www.github.com/raw-lab/metacerberus/issue)); we will fully annotate your genome, add

232 suggested pathways/metabolisms of interest, make custom HMMs to be added to  
233 MetaCerberus and FunGene.

234

## 235 **Results**

### 236 *Database size and download time*

237       Formatting and downloading are required steps in functional annotation and both  
238 depend on database size. Substantial databases take up large amounts of costly disk  
239 space and require expensive computers with large amounts of costly RAM for analysis.

240 MetaCerberus database size is 3.8 GB, with a download time of ~4 mins, and database  
241 format time is zero because they are pre-formatted already for the user (**Table 2**).

242 DRAM database download requires 710 GB of disc space, and requires ~3 days to  
243 download completely (**Table 2**). According to the DRAM readme, KEGG Genes and  
244 UniRef90 need ~500 GB of disc space and ~512 GB of RAM to process the complete  
245 database (**19**, <https://github.com/WrightonLabCSU/DRAM>). This database size difference is  
246 due UniRef90 updates since their original release in 2020. DRAM can run with more  
247 processors within a single node but is not set up for multi-node like MetaCerberus. The  
248 InterProScan database is 14 GB, which took ~2.45 h to install (**Table 2**). PROKKA had  
249 the smallest database at 636 MB and had the fastest install of ~3 ½ minutes (**Table 2**).  
250 MicrobeAnnotator requires at least ~237 GB for its full version and ~0.65 GB for its light  
251 version (**Table 2, 22**).

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255 *Computational resource comparison*

256 We compared MetaCerberus to DRAM, InterProScan, and PROKKA for the time  
257 used per genome, RAM utilization, and disk space used across 100 randomly selected  
258 bacterial genomes within GTDB (**Table S1, Fig 2**). Generally, PROKKA had the highest  
259 processing speed per genome (~48 sec median, **Fig 2**). InterProScan had the slowest  
260 at ~21 min per genome median time (**Fig 2**). DRAM was ~5 mins faster per genome  
261 than MetaCerberus (i.e., 10 mins vs 15 mins) (**Fig 2**). MetaCerberus and PROKKA had  
262 the lowest RAM, followed by InterProScan (**Fig 2**). DRAM using default parameters had  
263 the highest RAM observed (**Fig 2**). DRAM had the lowest disc space due to the deletion  
264 of files post-finalization, with PROKKA having the most disc space (**Fig 2**). EggNOG-  
265 mapper using HMMs was initially compared to MetaCerberus; however, further testing  
266 was not completed due to the high run time failure rate. On average, EggNOG-mapper  
267 failed to finish annotation 32% of the time using 148 randomly selected GTDB  
268 bacterial/archaeal genomes used by other tools (**Table S3**). Approximately 16% of the  
269 genomes failed after running for two days; another 16% could not annotate even when  
270 other tools, including MetaCerberus, had no issue (**Table S3**). The average annotation  
271 time with EggNOG-mapper v2 was ~53 min, with a median of ~30 min (**Table S3**). It  
272 was the slowest tool tested and thus removed from further comparisons.

273 MicrobeAnnotator didn't functionally install the database correctly. We could not  
274 successfully use the code; thus, it was removed from further comparisons..

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278 *Automatic statistical and pathway analysis*

279       MetaCerberus provides automatic differential statistics, pathway gene  
280    enrichments, and KEGG map-based heatmaps in Pathview R for data exploration, data  
281    mining, and hypothesis generation. As a test, we compared five distinct genospecies,  
282    *Rhizobium leguminosarum*, against five distinct *Exiguobacterium* spp. using  
283    MetaCerberus using both DESeq2 and edgeR (**Table S3**). These genomes were  
284    selected as a comparison due to differential pathways within the comparison genomes.  
285    *Rhizobium* are symbiotic nitrogen fixers containing both nitrogenase for nitrogen fixation  
286    and nodulation genes for symbiotic nodule formation within legume roots (**53**). The  
287    *Exiguobacterium* spp. Have a bright orange colony morphology color from  
288    biosynthetically made carotenoids; it is hypothesized that the carotenoid  
289    diaponeurosporene-4-oic acid from the C<sub>30</sub> carotenoid biosynthesis pathway is what  
290    provides the distinctive orange color (**54**). Chemical studies have suggested other  
291    carotenoids are present within *Exiguobacterium* spp. that contribute to the orange  
292    colony color (**55**). MetaCerberus found differential pathway assignments using DESeq2  
293    and Pathview for carotenoid biosynthesis, ABC transporters, and phosphotransferase  
294    system (including nitrogen regulation) (**Fig S2-4**). edgeR found an additional pathway in  
295    benzoate degradation that wasn't found in DESeq2 (**Fig S5**).

296

297 *Annotation comparisons*

298       PROKKA, DRAM, and MetaCerberus all use Prodigal for pORF calling.  
299    MetaCerberus also provides an extra pORF caller FragGeneScanRs. InterProScan  
300    uses the EMBOSS getorf pORF caller, which in all cases had lower pORFs than

301 Prodigal regardless of the genome kingdom type (e.g., bacteria, archaea, CPR, phage,  
302 archaeal virus or eukaryotic virus) (**Fig S6**). Generally, PROKKA, DRAM, and  
303 MetaCerberus had similar pORF calling numbers; however, DRAM did call more pORF  
304 from eukaryotic viruses (**Fig S6**).

305 Furthermore, we compared MetaCerberus to DRAM, InterProScan, and  
306 PROKKA for whether a pORF was annotated, listed as hypothetical, or unknown (no  
307 annotation). We randomly selected 100 unique bacterial genomes from GTDB, 100  
308 unique archaea genomes from GTDB, 100 unique phage genomes from INPHARED,  
309 100 unique eukaryotic viral genomes from RefSeq, 78 CPR genomes, and 82 archaeal  
310 viral genomes for these annotation tests (**Table S1**). MetaCerberus, DRAM, and  
311 InterProScan protein modes had similar annotation results of ~78-83% for bacteria, with  
312 InterProScan being the highest at 83% (**Fig 3**). InterProScan using nucleotide mode  
313 had the lowest annotation amount across all kingdoms (**Fig 3-4**). PROKKA had ~50% of  
314 the pORFs as annotated and hypothetical for bacteria and ~60% hypothetical for  
315 archaea (**Fig 3**). CPR annotation InterProScan had the highest at 70%, followed by  
316 DRAM at 66%, then MetaCerberus at 61% (**Fig 3**). MetaCerberus and PROKKA had  
317 fewer unknowns than DRAM for bacteria, archaea, and CPR genomes (**Fig 3**).  
318 PROKKA annotated very few CPR pORFs, with the majority >60% being hypothetical  
319 proteins (**Fig 3**). DRAM generally doesn't find many hypothetical proteins or lists them  
320 as unknown across domains of the tree of life.

321 MetaCerberus performs better for viruses, phages, and archaeal viruses (**Fig 4**).  
322 MetaCerberus annotates more per genome >63 % phages, >65 % viruses, and >41%  
323 archaeal viruses based on median values (**Fig 4**). MetaCerberus outperforms across all

324 viruses (e.g., phages, eukaryotic viruses, and archaeal viruses) providing more  
325 annotations, fewer hypotheticals, and fewer unknowns compared to DRAM,  
326 InterProScan, and PROKKA (**Fig 4**). MetaCerberus annotates more KOs from KOfams  
327 than DRAM across all domains (**Fig 5**). PROKKA and InterProScan don't provide KOs;  
328 therefore, we couldn't compare KOs found across domains to MetaCerberus.

329 To better compare to DRAM-v, the only other tool exclusively for viruses and  
330 phages, we analyzed a virome containing 1,907 viral populations (VPs) obtained from  
331 Swedish permafrost. Based on time, MetaCerberus took 99 mins to complete the  
332 annotation compared to 141.75 mins for DRAM (**Fig 6**). When MetaCerberus is utilized  
333 at its full potential with RAY it only takes 12.5 mins for the same dataset (**Fig 6**). RAM  
334 was significant less with MetaCerberus vs. DRAM-v, both in MPP and non-MPP mode,  
335 with <500 Mb of RAM compared to 18.7 Gb with DRAM-v (**Fig 6**). MetaCerberus had  
336 more annotations than DRAM-v for the Swedish permafrost virome across shared  
337 databases (i.e., KO, CAZy, and VOG) (**Fig S7**).

338

### 339 **Discussion**

340 MetaCerberus provides a low memory, robust, scalable, and rapid annotation  
341 across the tree of life, exclusively using HMMs/HMMER. HMMER is a powerful tool to  
342 find pORFs that may be missed by standard homology-based tools due to its protein  
343 domain centric and supervised machine-learning nature. It's rarely used alone due to  
344 the speed and time required to finish annotation. MetaCerberus has provided a solution  
345 to this scaling issue using RAY and algorithms needed from MerCat2. EggNOG-Mapper  
346 v2 is the only other tool that exclusively provides HMMs/HMMER-based annotation

347 alone. MetaCerberus runs twice as fast on a single node than EggNOG-Mapper v2  
348 without RAY. MetaCerberus with RAY is three times as fast as EggNOG-Mapper v2  
349 (data not shown) on a database that is 1/3 smaller.

350 Generally, MetaCerberus performs better for viral and phage annotation when  
351 directly compared to DRAM-v. DRAM finds more pORFs than MetaCerberus (**Fig S6**)  
352 due to it using the -meta option in Prodigal for viruses; whereas, MetaCerberus uses a  
353 standard for complete genomes in this case but still can annotate viral genomes better  
354 than DRAM on a much smaller database. Viruses, archaeal viruses, and phages are a  
355 grand challenge to unlock the 'unknown' and 'hypothetical' functions within their  
356 genomes.

357 As data scales, computational time, memory, and waiting for results will take  
358 longer. Scalable tools like MetaCerberus are needed as we approach Petabyte levels of  
359 sequencing. MetaCerberus provides a further community resource to annotate the  
360 unknowns of our biosphere. Lastly, MetaCerberus provides a robust tool kit to annotate  
361 the entire tree of life at scale.

362

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374

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681 **Figure and Table Legends**

682 **Table 1:** Comparing tools based on databases provided. This includes versions of other  
683 databases present within the various tools compared.

684

685 **Table 2:** Database size, download, and formatting time across tools.

686

687 **Figure 1:** Flowgraph of the MetaCerberus pipeline. MetaCerberus has three input data  
688 formats which include raw reads, contigs, or previously called pORFs. Quality control  
689 step is mainly utilized for raw reads only. Contigs have a formatting mode where they are  
690 quality controlled for N presence, followed by N removal, and also provides basic contig  
691 statistics using Metaome Stats (e.g., N50, N90 etc). Gene calling currently offers  
692 prodigal or FragGeneScanRs for pORF calling. Gene prediction is completed with  
693 HMMER/HMM against KEGG and FOAM KOs (all by default). Users can select  
694 additional databases such as CAZy, COG, PHROG, VOG, and pVOGs for viruses, or  
695 selective KOfams for prokaryotes or eukaryotes. With running four or more samples it  
696 provides a PCA for KEGG and FOAM KOs, a basic run metric dashboard, as well as  
697 differential statistics using DESeq2/edgeR, and pathway enrichment using GAGE R  
698 followed by plotting in Pathview R.

699

700 **Figure 2:** Computational resource comparison. DRAM, InterProScan, PROKKA, and  
701 MetaCerberus are compared computationally for time to complete each genome  
702 annotation, RAM required to complete annotation per genome, and disc space needed  
703 for inputs/outputs. The 100 randomly selected bacterial genomes were from GTDB  
704 (**Table S1**).

705

706 **Figure 3:** Annotation comparison across cellular domains of life (bacteria, archaea,  
707 CPR). MetaCerberus was compared to DRAM, InterProScan, and PROKKA for  
708 annotation across various genomes. Supplemental materials include the genomes for  
709 bacteria, archaea, and CPR used in this comparison (**Table S1**).

710

711 **Figure 4:** Annotation comparison across viruses infecting differential cellular domains  
712 (phage, archaeal viruses, eukaryotic viruses). MetaCerberus was compared to DRAM,  
713 InterProScan, and PROKKA for annotation across various genomes. The genomes are  
714 listed for phage, archaeal viruses, and eukaryotic viruses in supplemental materials  
715 (**Table S1**).

716

717 **Figure 5.** DRAM vs. MetaCerberus KO annotation comparison across the domains of  
718 life. DRAM and MetaCerberus utilize KOfams for KEGG KO assignment if the user  
719 doesn't provide a KEGG KO database separately. The genomes for the comparison are  
720 listed in supplemental materials (**Table S1**). The e-values and bitscore can vary  
721 between DRAM and MetaCerberus. In this comparison, we choose the default dbCAN  
722 e-value option of  $<1e^{-15}$  and the default bitscore of 60 for DRAM.

723

724 **Figure 6.** DRAM vs. MetaCerberus computational resource comparison. A virome from  
725 Swedish permafrost containing 1,907 VPs were compared computationally for time to

726 complete annotation, RAM required to complete annotation, and disc space needed for  
727 inputs/outputs. MPP testing for MetaCerberus utilized five nodes for comparisons.  
728

## 729 **Supplemental Materials**

730

731 **Table S1:** List of genomes used for computational comparisons. This includes  
732 randomly selected GTDB genomes for archaea and bacteria domains, phage genomes,  
733 archaeal viral genomes, CPR genomes, and RefSeq viral genomes.  
734

735 **Figure S1:** Standard output dashboard for MetaCerberus. Outputs include a complete  
736 html drawn in plotly. Also, for comparisons of >3 genomes, FOAM and KEGG-based KO  
737 PCA are included.  
738

739 **Figure S2:** Comparisons *Rhizobium* vs. *Exiguobacterium* genomes using MetaCerberus  
740 for carotenoid pathways in Pathview. KO counts from KEGG were normalized with  
741 DESeq2, enriched with GAGE, then plotted with Pathview R. Genomes are listed in  
742 supplemental materials (**Table S1**).  
743

744 **Figure S3:** Comparisons *Rhizobium* vs. *Exiguobacterium* genomes using MetaCerberus  
745 for ABC transporters in Pathview. KO counts from KEGG were normalized with  
746 DESeq2, enriched with GAGE, then plotted with Pathview R. Genomes are listed in  
747 supplemental materials (**Table S1**).  
748

749 **Figure S4:** Comparisons *Rhizobium* vs. *Exiguobacterium* genomes using MetaCerberus  
750 for phosphotransferase system in Pathview. KO counts from KEGG were normalized with  
751 DESeq2, enriched with GAGE, then plotted with Pathview R. Genomes are listed in  
752 supplemental materials (**Table S1**).  
753

754 **Figure S5:** Comparisons *Rhizobium* vs. *Exiguobacterium* genomes using MetaCerberus  
755 for Benzoate degradation in Pathview. KO counts from KEGG was normalized with  
756 edgeR, enriched with GAGE, then plotted with Pathview R. Genomes are listed in  
757 supplemental materials (**Table S1**).  
758

759 **Figure S6:** Comparisons of protein-coding open reading frame (pORF) calling across  
760 computational tools. All tools but InterProScan use Prodigal for pORFs. InterProScan  
761 uses the EMBOSS getorf tool.  
762

763 **Figure S7:** Comparing DRAM-v vs. MetaCerberus for annotation across shared  
764 databases (i.e., KO, VOG, CAZy). The Swedish virome was utilized for comparisons.  
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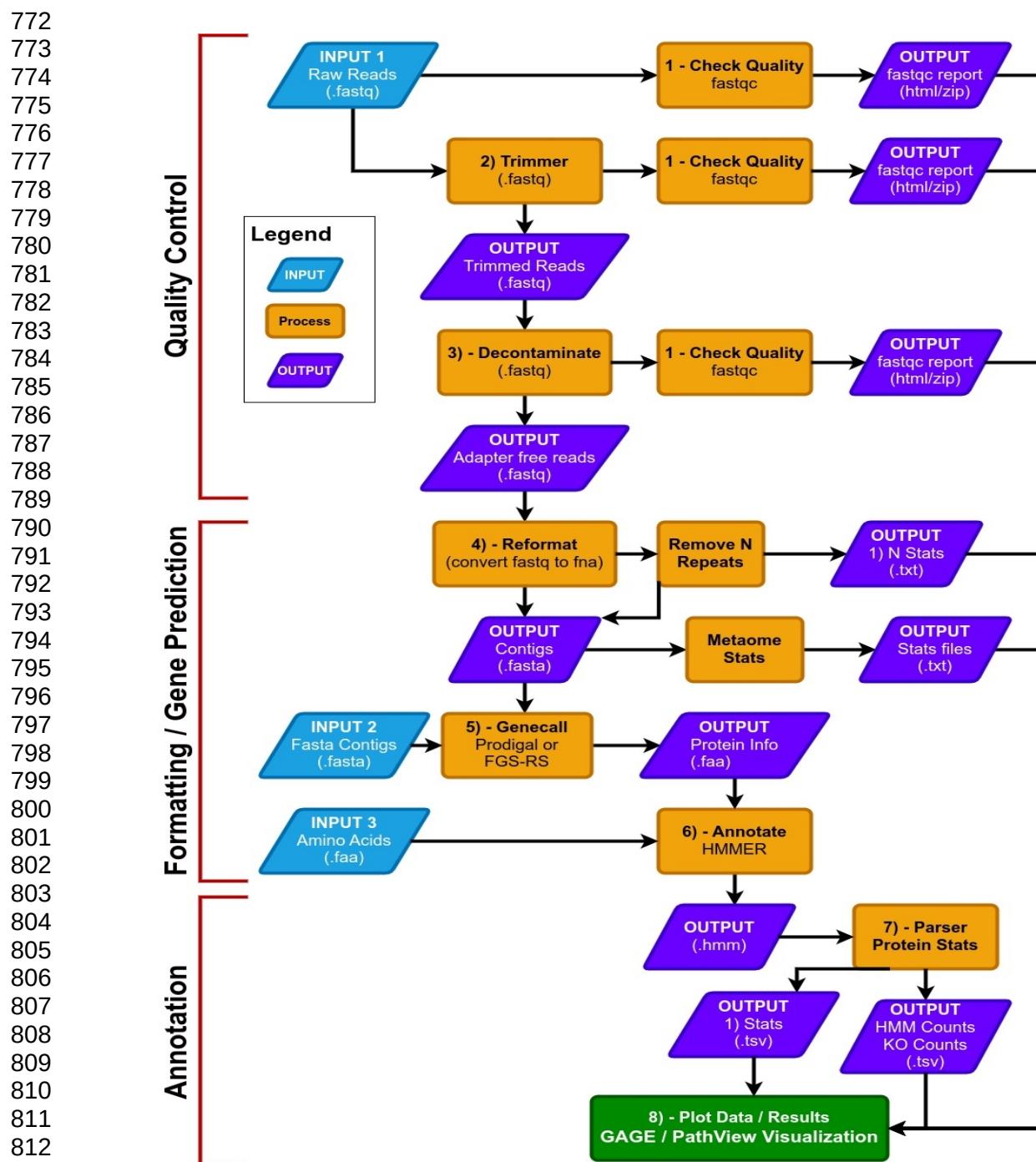
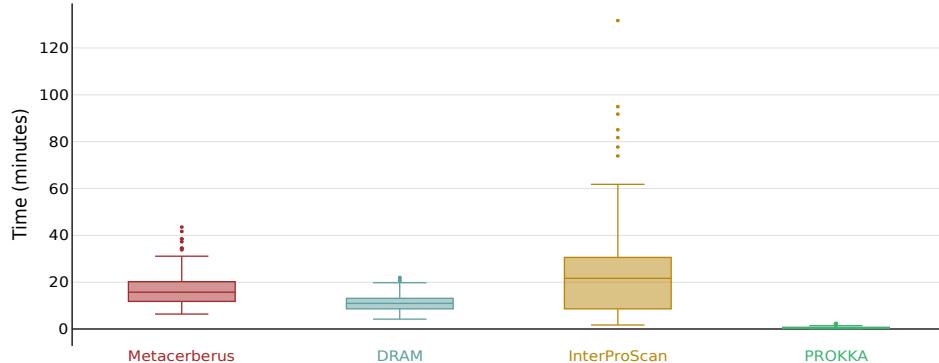


Figure 1.

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### GTDB-bacteria

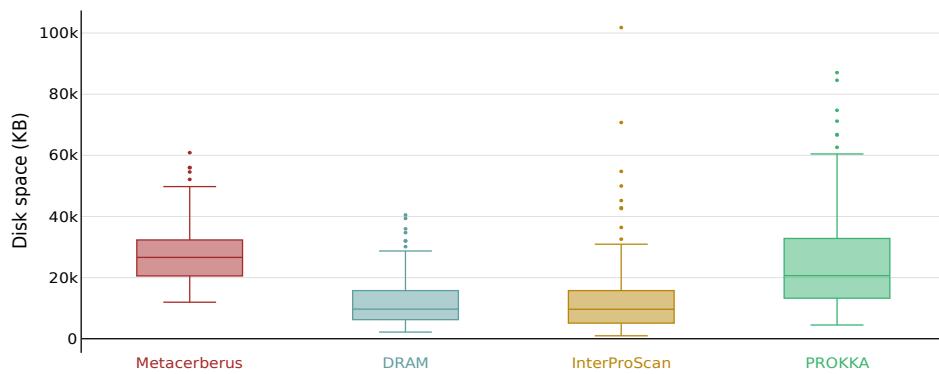
#### A) Time spent



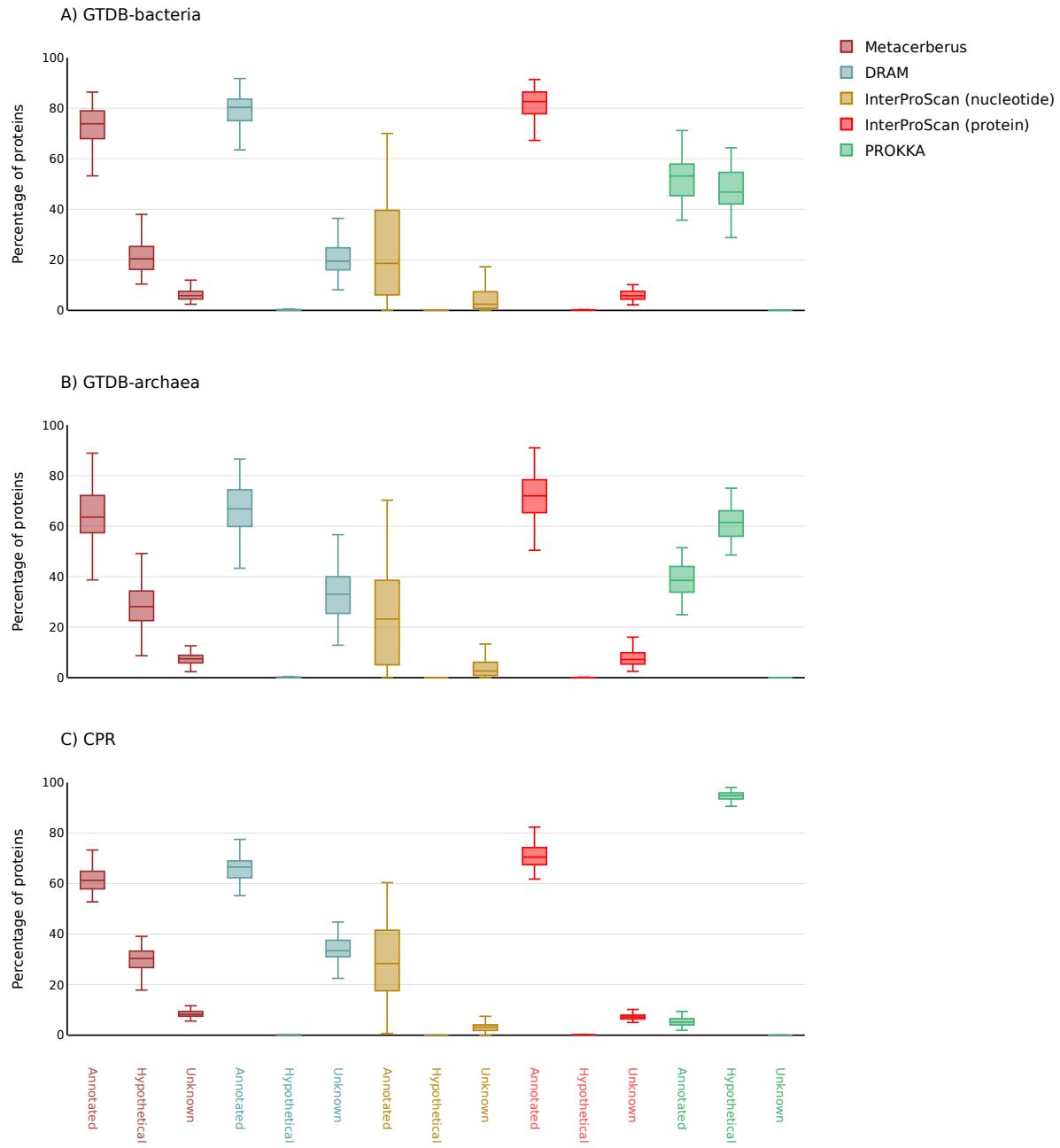
#### B) RAM used



#### C) Disk used



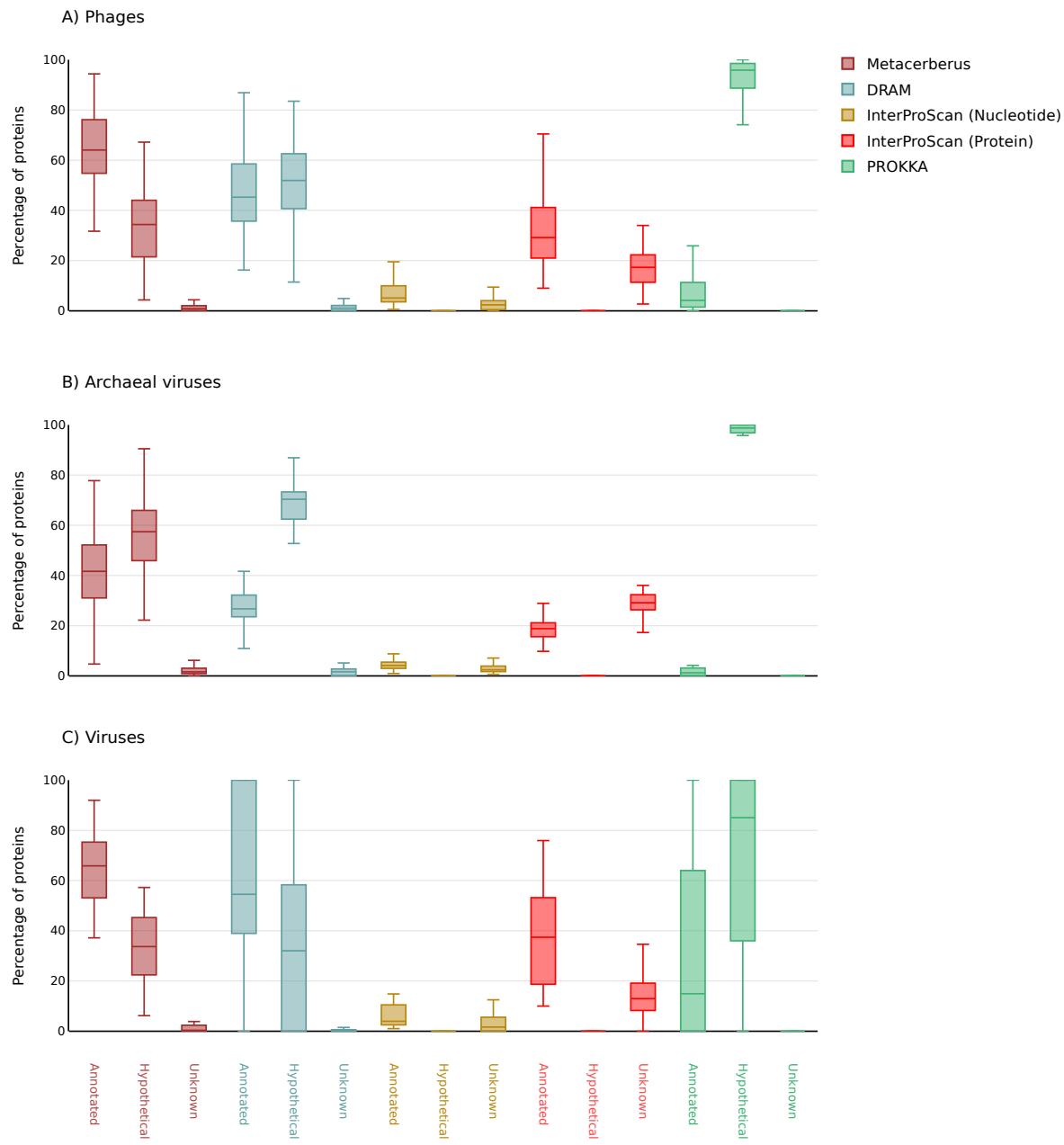
**Figure 2.**



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**Figure 3.**

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886 **Figure 4.**

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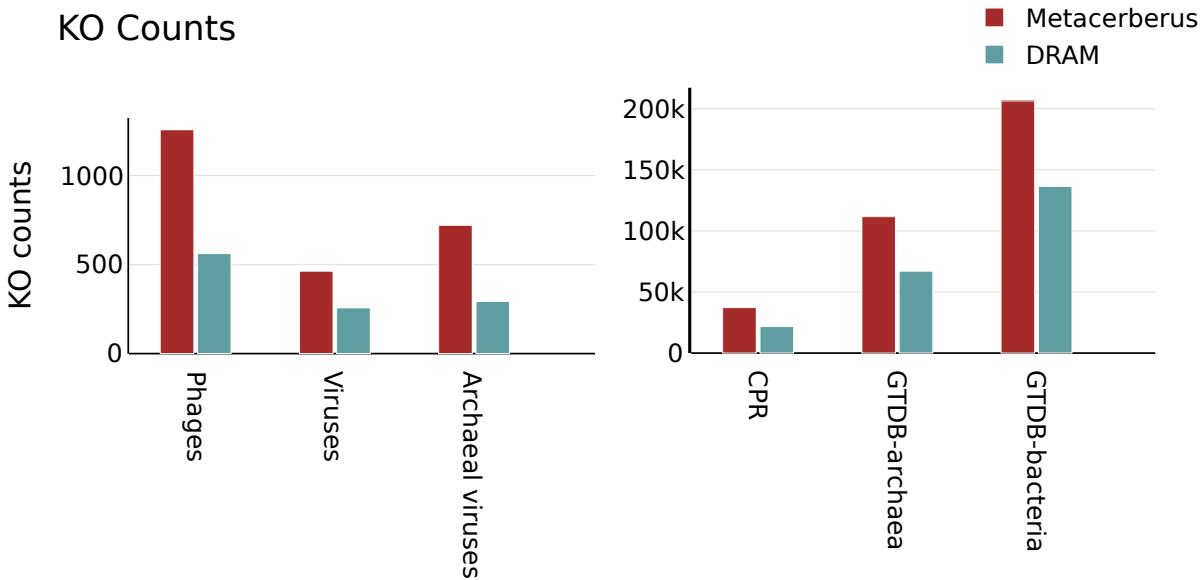
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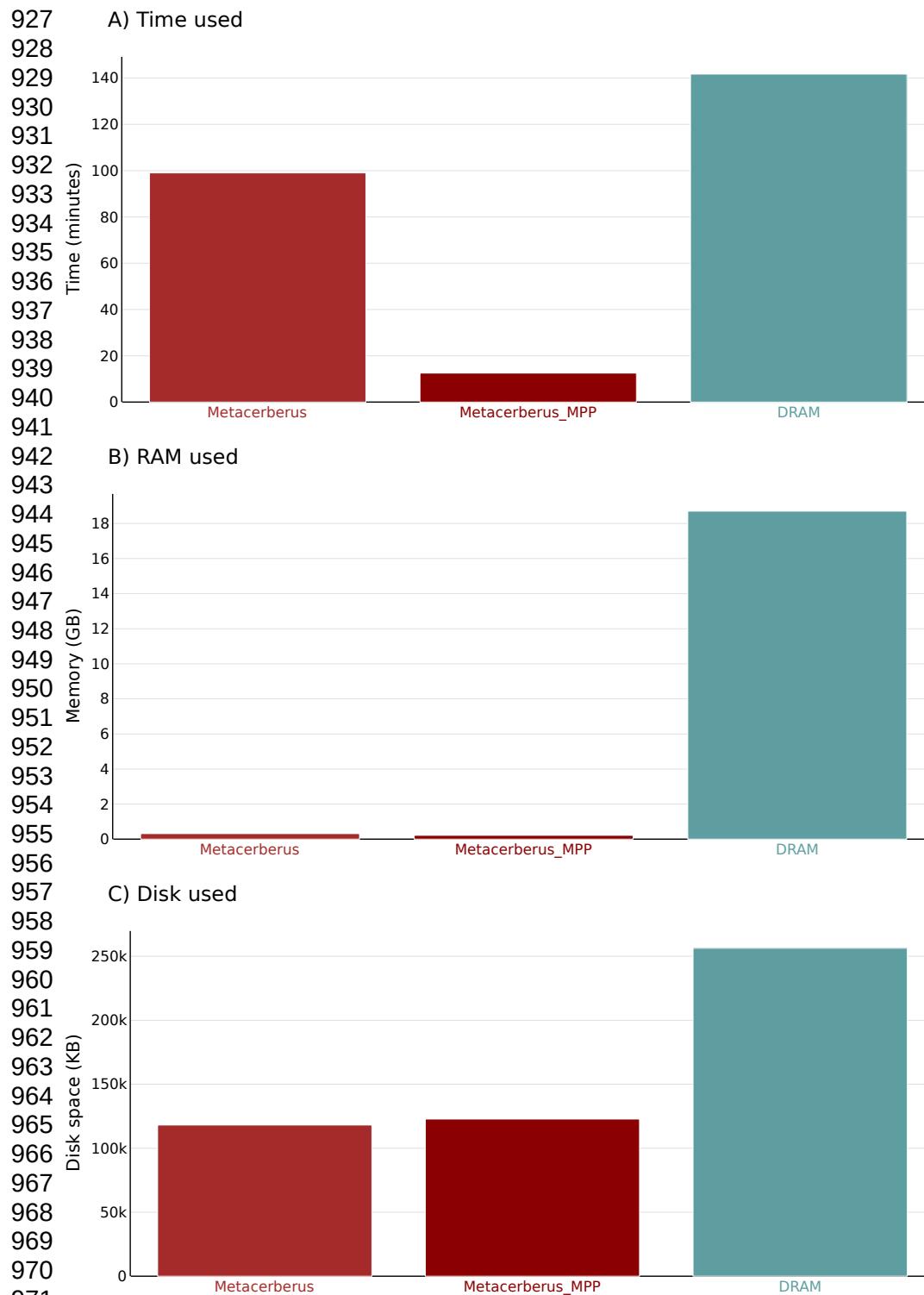
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897 **Figure 5.**

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978 **Table 1**

	EC	KEGG	CAZy	COG	FOAM	VOG	pVOG	PHROG	pfam	EggNOG	InterPro
MetaCerberus	X	X	X	X	X	X	X	X			
DRAM	X	X	X			X			X		
Prokka	X								X		
InterProScan	X								X		X
MicrobeAnnotator	X	X									X
EggNOG-Mapper	X	X	X	X					X	X	

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**Table 2**

Tool	Time	Disk	Version
DRAM	~ 3 days	~710GB	v1.4.6
InterProScan	~ 2:45:59.23	14GB	v5.60-92.0
Metacerberus	~ 0:04:14.29	3.8GB	v1.1
PROKKA	~ 0:03:28.68	607M	v1.14.6
EggNOG-Mapper	~14:33:31.74	31GB	V2.1.8
MicrobeAnnotator	>3 days	~237GB	v2.0.5

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