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A benchmark of metagenomics software

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Critical Assessment of Metagenome Interpretation—a benchmark of metagenomics software

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Methods for assembly, taxonomic profiling and binning are key to interpreting metagenome data, but a lack of consensus about benchmarking complicates performance assessment. The Critical Assessment of Metagenome Interpretation (CAMI) challenge has engaged the global developer community to benchmark their programs on highly complex and realistic data sets, generated from ~700 newly sequenced microorganisms and ~600 novel viruses and plasmids and representing common experimental setups. Assembly and genome binning programs performed well for species represented by individual genomes but were substantially affected by the presence of related strains. Taxonomic profiling and binning programs were proficient at high taxonomic ranks, with a notable performance decrease below family level. Parameter settings markedly affected performance, underscoring their importance for program reproducibility. The CAMI results highlight current challenges but also provide a roadmap for software selection to answer specific research questions.

The biological interpretation of metagenomes relies on sophisticated computational analyses such as read assembly, binning and taxonomic profiling. Tremendous progress has been achieved¹, but there is still much room for improvement. The evaluation of computational methods has been limited largely to publications presenting novel or improved tools. These results are extremely difficult to compare owing to varying evaluation strategies, benchmark data sets and performance criteria. Furthermore, the

state of the art in this active field is a moving target, and the assessment of new algorithms by individual researchers consumes substantial time and computational resources and may introduce unintended biases.

We tackle these challenges with a community-driven initiative for the Critical Assessment of Metagenome Interpretation (CAMI). CAMI aims to evaluate methods for metagenome analysis comprehensively and objectively by establishing standards through community involvement in the design of benchmark data sets, evaluation procedures, choice of performance metrics and questions to focus on. To generate a comprehensive overview, we organized a benchmarking challenge on data sets of unprecedented complexity and degree of realism. Although benchmarking has been performed before^{2,3}, this is the first community-driven effort that we know of. The CAMI portal is also open to submissions, and the benchmarks generated here can be used to assess and develop future work.

We assessed the performance of metagenome assembly, binning and taxonomic profiling programs when encountering major challenges commonly observed in metagenomics. For instance, microbiome research benefits from the recovery of genomes for individual strains from metagenomes^{4–7}, and many ecosystems have a high degree of strain heterogeneity^{8,9}. To date, it is not clear how much assembly, binning and profiling software are influenced by the evolutionary relatedness of organisms, community complexity, presence of poorly categorized taxonomic groups (such as viruses) or varying software parameters.

A full list of affiliations appears at the end of the paper.

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RESULTS

We generated extensive metagenome benchmark data sets from newly sequenced genomes of ~700 microbial isolates and 600 circular elements that were distinct from strains, species, genera or orders represented by public genomes during the challenge. The data sets mimicked commonly used experimental settings and properties of real data sets, such as the presence of multiple, closely related strains, plasmid and viral sequences and realistic abundance profiles. For reproducibility, CAMI challenge participants were encouraged to provide predictions along with an executable Docker biobox¹⁰ implementing their software and specifying the parameter settings and reference databases used. Overall, 215 submissions, representing 25 programs and 36 biobox implementations, were received from 16 teams worldwide, with consent to publish (Online Methods).

Assembly challenge

Assembling genomes from metagenomic short-read data is very challenging owing to the complexity and diversity of microbial communities and the fact that closely related genomes may represent genome-sized approximate repeats. Nevertheless, sequence assembly is a crucial part of metagenome analysis, and subsequent analyses—such as binning—depend on the assembly quality.

Overall performance trends

Developers submitted reproducible results for six assemblers: MEGAHIT¹¹, Minia¹², Meraga (Meraculous¹³ + MEGAHIT), A* (using the OperaMS Scaffold¹⁴), Ray Meta¹⁵ and Velour¹⁶. Several are dedicated metagenome assemblers, while others are more broadly used (Supplementary Tables 1 and 2). Across all data sets (Supplementary Table 3) the assembly statistics (Online Methods) varied substantially by program and parameter settings (Supplementary Figs. 1–12). The gold-standard co-assembly of the five samples constituting the high-complexity data set has 2.80 Gbp in 39,140 contigs. The assembly results ranged from 12.32 Mbp to 1.97 Gbp in size (0.4% and 70% of the gold standard co-assembly, respectively), 0.4% to 69.4% genome fraction, 11 to 8,831 misassemblies and 249 bp to 40.1 Mbp unaligned contigs (Supplementary Table 4 and Supplementary Fig. 1). MEGAHIT¹¹ produced the largest assembly, of 1.97 Gbp, with 587,607 contigs, 69.3% genome fraction and 96.9% mapped reads. It had a substantial number of unaligned bases (2.28 Mbp) and the most misassemblies (8,831). Changing the parameters of MEGAHIT (*Megahit_ep_mtl200*) substantially increased the unaligned bases, to 40.89 Mbp, whereas the total assembly length, genome fraction and fraction of mapped reads remained almost identical (1.94 Gbp, 67.3% and 97.0%, respectively, with 7,538 misassemblies). Minia¹² generated the second largest assembly (1.85 Gbp in 574,094 contigs), with a genome fraction of 65.7%, only 0.12 Mbp of unaligned bases and 1,555 misassemblies. Of all reads, 88.1% mapped to the Minia assembly. Meraga generated an assembly of 1.81 Gbp in 745,109 contigs, to which 90.5% of reads mapped (2.6 Mbp unaligned, 64.0% genome fraction and 2,334 misassemblies). Velour (*VELOUR_k63_C2.0*) produced the most contigs (842,405) in a 1.1-Gbp assembly (15.0% genome fraction), with 381 misassemblies and 56 kbp unaligned sequences. 81% of the reads mapped to the Velour assembly. The smallest assembly was produced by Ray⁶ using *k*-mer of 91 (*Ray_k91*) with 12.3 Mbp

assembled into 13,847 contigs (genome fraction <0.1%). Only 3.2% of the reads mapped to this assembly.

Altogether, MEGAHIT, Minia and Meraga produced results of similar quality when considering these various metrics; they generated a higher contiguity than the other assemblers (Supplementary Figs. 10–12) and assembled a substantial fraction of genomes across a broad abundance range. Analysis of the low- and medium-complexity data sets delivered similar results (Supplementary Figs. 4–9).

Closely related genomes

To assess how the presence of closely related genomes affects assemblies, we divided genomes according to their average nucleotide identity (ANI)¹⁷ into ‘unique strains’ (genomes with <95% ANI to any other genome) and ‘common strains’ (genomes with an ANI ≥95% to another genome in the data set). Meraga, MEGAHIT and Minia recovered the largest fraction of all genomes (Fig. 1a). For unique strains, Minia and MEGAHIT recovered the highest percentages (median over all genomes 98.2%), followed by Meraga (median 96%) and *VELOUR_k31_C2.0* (median 62.9%) (Fig. 1b). Notably, for the common strains, all assemblers recovered a substantially lower fraction (Fig. 1c). MEGAHIT (*Megahit_ep_mtl200*; median 22.5%) was followed by Meraga (median 12.0%) and Minia (median 11.6%), whereas *VELOUR_k31_C2.0* recovered only 4.1% (median). Thus, the metagenome assemblers produced high-quality results for genomes without close relatives, while only a small fraction of the common strain genomes was assembled, with assembler-specific differences. For very high ANI groups (>99.9%), most assemblers recovered single genomes (Supplementary Fig. 13). Resolving strain-level diversity posed a substantial challenge to all programs evaluated.

Effect of sequencing depth

To investigate the effect of sequencing depth on the assemblies, we compared the genome recovery rate (genome fraction) to the genome sequencing coverage (Fig. 1d and Supplementary Fig. 2 for complete results). Assemblers using multiple *k*-mers (Minia, MEGAHIT and Meraga) substantially outperformed single *k*-mer assemblers. The chosen *k*-mer size affects the recovery rate (Supplementary Fig. 3): while small *k*-mers improved recovery of low-abundance genomes, large *k*-mers led to a better recovery of highly abundant ones. Most assemblers except for Meraga and Minia did not recover very-high-copy circular elements (sequencing coverage >100×) well, though Minia lost all genomes with 80–200× sequencing coverage (Fig. 1d). Notably, no program investigated contig topology to determine whether these were circular and complete.

Binning challenge

Metagenome assembly programs return mixtures of variable-length fragments originating from individual genomes. Binning algorithms were devised to classify or bin these fragments—contigs or reads—according to their genomic or taxonomic origins, ideally generating draft genomes (or pan-genomes) of a strain (or higher-ranking taxon) from a microbial community. While genome binners group sequences into unlabeled bins, taxonomic binners group the sequences into bins with a taxonomic label attached.

Results were submitted together with software bioboxes for five genome binners and four taxonomic binners: MyCC¹⁸, MaxBin

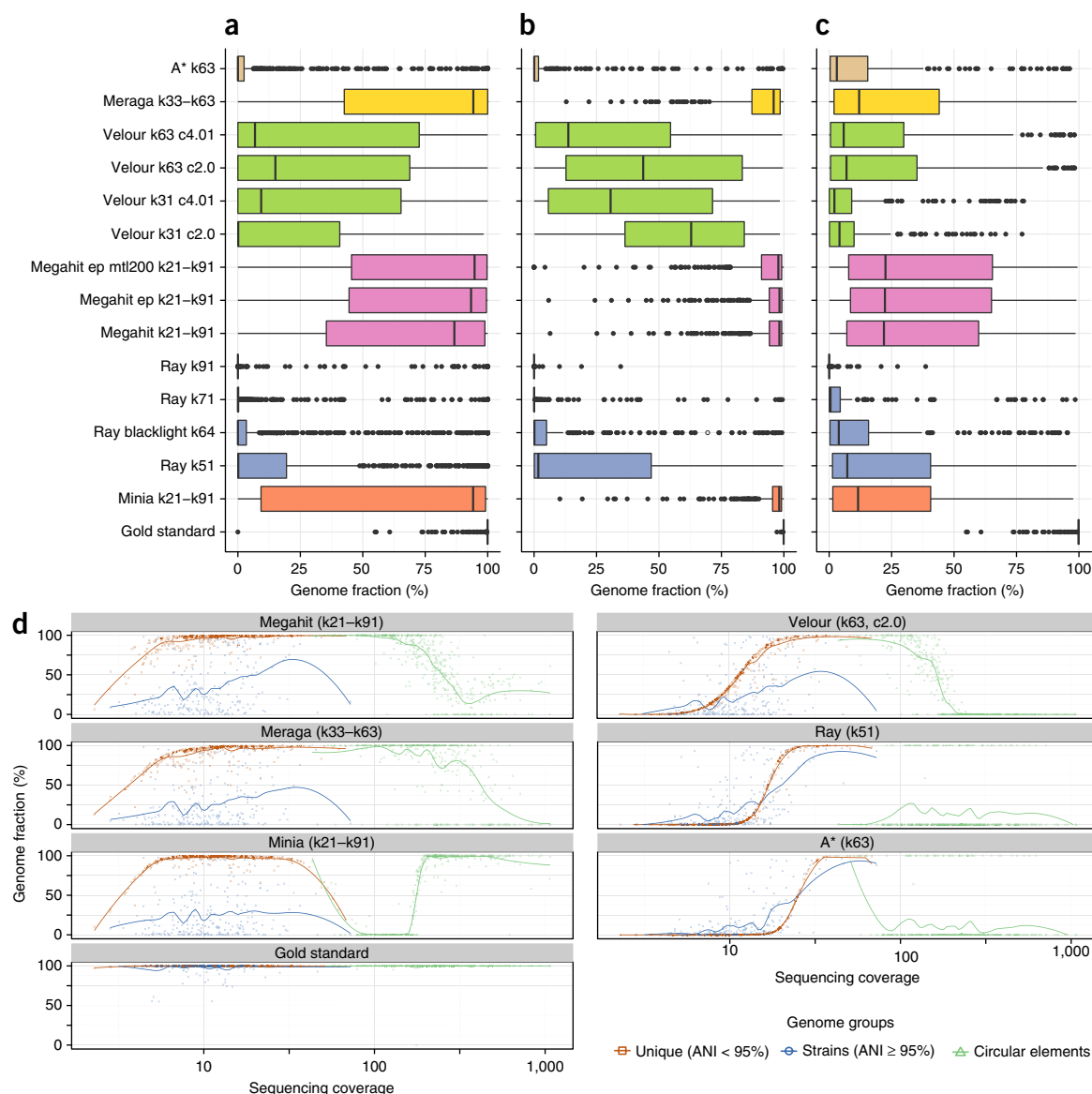


Figure 1 | Assembly results for the CAMI high-complexity data set. (a–c) Fractions of reference genomes assembled by each assembler for all genomes (a), genomes with ANI < 95% (b) and genomes with ANI ≥ 95% (c). Colors indicate results from the same assembler incorporated in different pipelines or parameter settings (see **Supplementary Table 2** for details). Dots indicate individual data points (genomes); boxes, interquartile range; center lines, median. (d) Genome recovery fraction versus genome sequencing depth (coverage). Data were classified as unique genomes (ANI < 95%, brown), genomes with related strains present (ANI ≥ 95%, blue) or high-copy circular elements (green). The gold standard includes all genomic regions covered by at least one read in the metagenome data set.

2.0 (ref. 19), MetaBAT²⁰, MetaWatt 3.5 (ref. 21), CONCOCT²², PhyloPythiaS+²³, taxator-tk²⁴, MEGAN6 (ref. 25) and Kraken²⁶. Submitters ran their programs on the gold-standard co-assemblies or on individual read samples (MEGAN6), according to their suggested application. We determined their performance for addressing important questions in microbiome studies.

Recovery of individual genome bins

We investigated program performance when recovering individual genome (strain-level) bins (Online Methods). For the genome binner, average genome completeness (34% to 80%) and purity (70% to 97%) varied substantially (**Supplementary Table 5** and **Supplementary Fig. 14**). For the medium- and low-complexity data sets, MaxBin 2.0 had the highest values (70–80%

completeness, >92% purity), followed by other programs with comparably good performance in a narrow range (completeness ranging with one exception from 50–64%, >75% purity). Notably, other programs assigned a larger portion of the data sets than MaxBin 2.0 measured in bp, though with lower adjusted Rand index (ARI; **Fig. 2a**). For applications where binning a larger fraction of the data set at the cost of some accuracy is important, MetaWatt 3.5, MetaBAT and CONCOCT could be good choices. The high-complexity data set was more challenging to all programs, with average completeness decreasing to ~50% and more than 70% purity, except for MaxBin 2.0 and MetaWatt 3.5, which showed purity of above 90%. The programs either assigned only a smaller data set portion (>50%, in the case of MaxBin 2.0) with high ARI or a larger fraction with lower ARI (more than 90% with less than 0.5 ARI, all except MaxBin and MetaBat).

The exception was MetaWatt 3.5, which assigned more than 90% of the data set with an ARI larger than 0.8, thus best recovering abundant genomes from the high-complexity data set. Accordingly, MetaWatt 3.5, followed by MaxBin 2.0, recovered the most genomes with high purity and completeness from all data sets (**Fig. 2b**).

Effect of strain diversity

For unique strains, the average purity and completeness per genome bin was higher for all genome binner (**Fig. 2c**). For the medium- and low-complexity data sets, all had a purity of above 80%, while completeness was more variable. MaxBin 2.0 performed best across all data sets, with more than 90% purity and completeness of 70% or higher. MetaBAT, CONCOCT and MetaWatt 3.5 performed almost as well for two data sets.

For the common strains, however, completeness decreased substantially (**Fig. 2d**), similarly to purity for most programs. MaxBin 2.0 still stood out, with more than 90% purity on all data sets. Notably, when we considered the value of taxon bins for genome reconstruction, taxon bins had lower completeness but reached a similar purity, thus delivering high-quality, partial genome bins (**Supplementary Note 1** and **Supplementary Fig. 15**). Overall, very high-quality genome bins were reconstructed with genome binning programs for unique strains, whereas the presence of closely related strains presented a notable hurdle.

Performance in taxonomic binning

We next investigated the performance of taxonomic binner in recovering taxon bins at different ranks (Online Methods). These results can be used for taxon-level evolutionary or functional pan-genome analyses and conversion into taxonomic profiles.

For the low-complexity data set, PhyloPythiaS+ had the highest sample assignment accuracy, average taxon bin completeness and purity, which were all above 75% from domain to family level. Kraken followed, with average completeness and accuracy still above 50% to the family level. However, purity was notably lower, owing mainly to prediction of many small false bins, which affects purity more than overall accuracy (**Supplementary Fig. 16**). Removing the smallest predicted bins (1% of the data set) increased purity for Kraken, MEGAN and, most strongly, for taxator-tk, for which it was close to 100% until order level, and above 75% until family level (**Supplementary Fig. 17**). Thus, small bins predicted by these programs are not reliable, but otherwise, high purity can be reached for higher ranks. Below the family level, all programs performed poorly, either assigning very little data (low completeness and accuracy, accompanied by a low misclassification rate) or assigning more, with substantial misclassification. Notably, Kraken and MEGAN performed similarly. These programs utilize different data properties (**Supplementary Table 1**) but rely on similar algorithms.

The results for the medium-complexity data set agreed qualitatively with those for the low-complexity data set, except that Kraken, MEGAN and taxator-tk performed better (**Fig. 2e**). With the smallest predicted bins removed, both Kraken and PhyloPythiaS+ reached above 75% for accuracy, with average completeness and purity until family rank (**Fig. 2f**). Similarly, taxator-tk showed an average purity of almost 75% even at genus level (almost 100% until order level), and MEGAN showed an average purity of more than 75% at order level while maintaining accuracy and average completeness of around 50%. The results of

high-purity taxonomic predictions can be combined with genome bins to enable their taxonomic labeling. The performances on the high-complexity data set were similar (**Supplementary Figs. 18 and 19**).

Analysis of low-abundance taxa

We determined which programs delivered high completeness for low-abundance taxa. This is relevant when screening for pathogens in diagnostic settings²⁷ or for metagenome studies of ancient DNA samples. Even though PhyloPythiaS+ and Kraken had high completeness until family rank (**Fig. 2e,f**), completeness degraded at lower ranks and for low-abundance bins (**Supplementary Fig. 20**), which are most relevant for these applications. It therefore remains a challenge to further improve predictive performance.

Deep branchers

Taxonomic binner commonly rely on comparisons to reference sequences for taxonomic assignment. To investigate the effect of increasing evolutionary distances between a query sequence and available genomes, we partitioned the challenge data sets by their taxonomic distances to public genomes as genomes of new strains, species, genus or family (**Supplementary Fig. 21**). For new strain genomes from sequenced species, all programs performed well, with generally high purity and, often, high completeness, or with characteristics also observed for other data sets (such as low completeness for taxator-tk). At increasing taxonomic distances to the reference, both purity and completeness for MEGAN and Kraken dropped substantially, while PhyloPythiaS+ decreased most notably in purity, and taxator-tk, in completeness. For genomes at larger taxonomic distances ('deep branchers'), PhyloPythiaS+ maintained the best purity and completeness.

Influence of plasmids and viruses

The presence of plasmid and viral sequences had almost no effect on binning performance. Although the copy numbers were high, in terms of sequence size, the fraction was small (<1.5%, **Supplementary Table 6**). Only Kraken and MEGAN made predictions for the viral fraction of the data or predicted viruses to be present, albeit with low purity (<30%) and completeness (<20%).

Profiling challenge

Taxonomic profilers predict the taxonomic identities and relative abundances of microbial community members from metagenome samples and are used to study the composition, diversity and dynamics of microbial communities in a variety of environments^{28–30}. In contrast to taxonomic binning, profiling does not assign individual sequences. In some use cases, such as identification of potentially pathogenic organisms, accurate determination of the presence or absence of a particular taxon is important. In comparative studies (such as quantifying the dynamics of a microbial community over an ecological gradient), accurately determining the relative abundance of organisms is paramount.

Challenge participants submitted results for ten profilers: CLARK³¹; Common Kmers (an early version of MetaPalette)³²; DUDes³³; FOCUS³⁴; MetaPhlAn 2.0 (ref. 35); MetaPhyler³⁶; mOTU³⁷; a combination of Quikr³⁸, ARK³⁹ and SEK⁴⁰ (abbreviated Quikr); Taxy-Pro⁴¹ and TIPP⁴². Some programs were submitted with multiple versions or different parameter settings, bringing the number of unique submissions to 20.

Performance trends

We employed commonly used metrics (Online Methods) to assess the quality of taxonomic profiling submissions with regard to the biological questions outlined above. The reconstruction

fidelity for all profilers varied markedly across metrics, taxonomic ranks and samples. Each had a unique error profile and different strengths and weaknesses (Fig. 3a,b), but the profilers fell into three categories: (i) profilers that correctly predicted

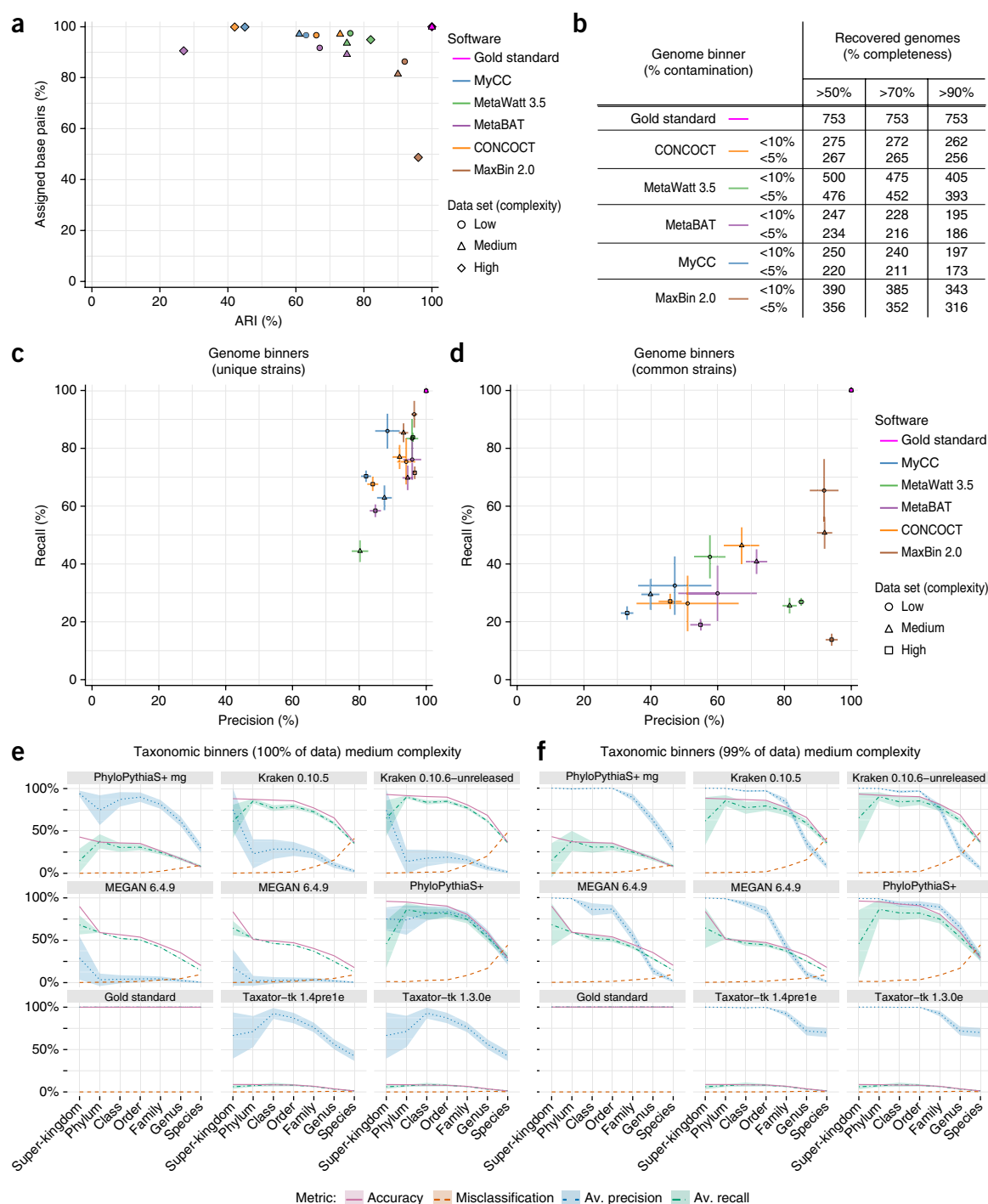


Figure 2 | Binning results for the CAMI data sets. (a) ARI in relation to the fraction of the sample assigned (in bp) by the genome binners. The ARI was calculated excluding unassigned sequences and thus reflects the assignment accuracy for the portion of the data assigned. (b) Number of genomes recovered with varying completeness and contamination (1-purity). (c,d) Average purity (precision) and completeness (recall) for genomes reconstructed by genome binners for genomes of unique strains with ANI <95% to others (c) and common strains with ANI ≥95% to each other (d). For each program and complexity data set (Supplementary Table 2), the submission with the largest sum of purity and completeness is shown. In each case, small bins adding up to 1% of the data set size were removed. Error bars, s.e.m. (e,f) Taxonomic binning performance metrics across ranks for the medium-complexity data set, with results for the complete data set (e) and smallest predicted bins summing up to 1% of the data set (f) removed. Shaded areas, s.e.m. in precision (purity) and recall (completeness) across taxon bins.

relative abundances, (ii) precise profilers and (iii) profilers with high recall. We quantified this with a global performance summary score (Online Methods, Fig. 3c, Supplementary Figs. 22–28 and Supplementary Table 7).

Quikr, CLARK, TIPP and Taxy-Pro had the highest recall, indicating their suitability for pathogen detection, where failure to identify an organism can have severe negative consequences. These were also among the least precise (Supplementary Figs. 29–33), typically owing to prediction of a large number of low-abundance organisms. MetaPhlAn 2.0 and Common Kmers were most precise, suggesting their use when many false positives would increase cost and effort in downstream analysis. MetaPhyler, FOCUS, TIPP, Taxy-Pro and CLARK best reconstructed relative abundances. On the basis of the average of precision and recall, over all samples and taxonomic ranks, Taxy-Pro version 0 (mean = 0.616), MetaPhlAn 2.0 (mean = 0.603) and DUDes version 0 (mean = 0.596) performed best.

Performance at different taxonomic ranks

Most profilers performed well only until the family level (Fig. 3a,b and Supplementary Figs. 29–33). Over all samples and programs at the phylum level, recall was 0.85 ± 0.19 (mean \pm s.d.), and L1 norm, assessing abundance estimate quality at a particular rank, was 0.38 ± 0.28 , both close to these metrics' optimal values (ranging from 1 to 0 and from 0 to 2, respectively), whereas precision was highly variable, at 0.53 ± 0.55 . Precision and recall were high for several methods (DUDes, Common Kmers, mOTU and MetaPhlAn 2.0) until order rank. However, accurately reconstructing a taxonomic profile is still difficult below family level. Even for the low-complexity sample, only MetaPhlAn 2.0 maintained its precision at species level, while the largest recall at genus rank for the low-complexity sample was 0.55, for Quikr. Across all profilers and samples, there was a drastic decrease in average performance between the family and genus levels, of $0.48 \pm 0.15\%$ and $0.52 \pm 0.18\%$ for recall and precision, respectively, but comparatively little change between order and family levels, with a decrease of only $0.1 \pm 0.07\%$ and $0.1 \pm 0.26\%$, for recall and precision, respectively. The other error metrics showed similar trends for all samples and methods (Fig. 3a and Supplementary Figs. 34–38).

Parameter settings and software versions

Several profilers were submitted with multiple parameter settings or versions (Supplementary Table 2). For some, this had little effect: for instance, the variance in recall among seven versions of FOCUS on the low-complexity sample at family level was only 0.002. For others, this caused large performance changes: for instance, one version of DUDes had twice the recall as that of another at phylum level on the pooled high-complexity sample (Supplementary Figs. 34–38). Notably, several developers submitted no results beyond a fixed taxonomic rank, as was the case for Taxy-Pro and Quikr. These submissions performed better than default program versions submitted by the CAMI team—indicating, not surprisingly, that experts can generate better results.

Performance for viruses and plasmids

We investigated the effect of including plasmids, viruses and other circular elements (Supplementary Table 6) in the gold-standard taxonomic profile (Supplementary Figs. 39–41). Here, the term

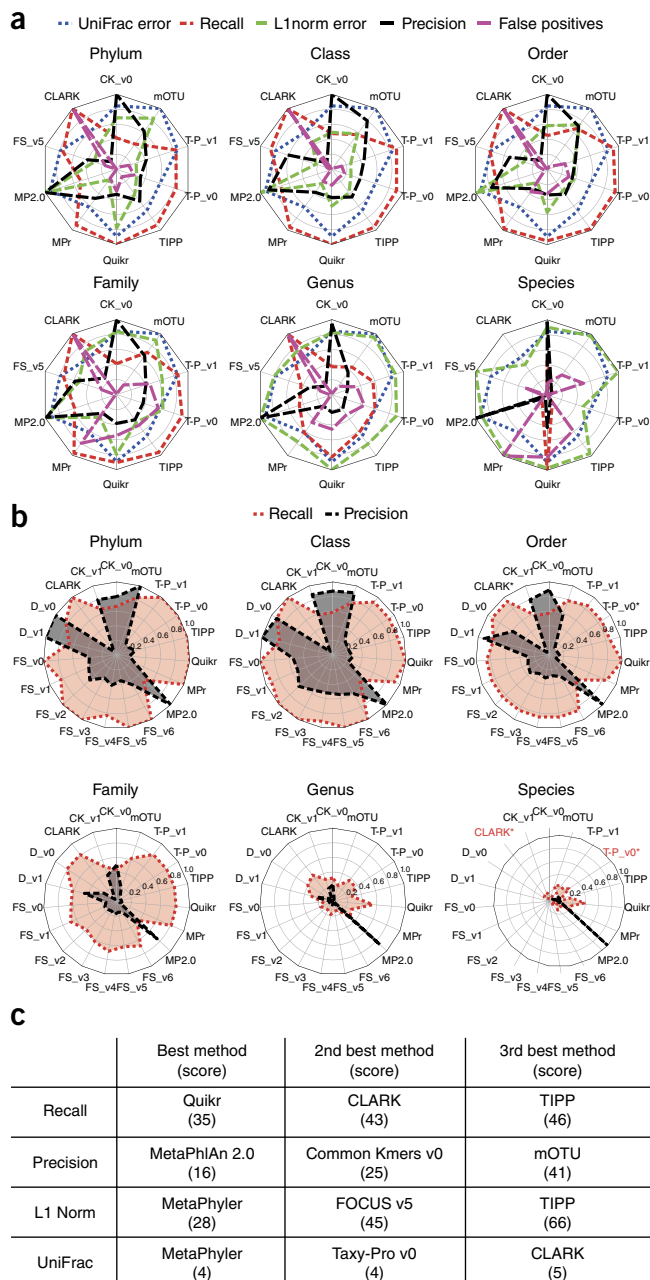


Figure 3 | Profiling results for the CAMI data sets. **(a)** Relative performance of profilers for different ranks and with different error metrics (weighted UniFrac, L1 norm, recall, precision and false positives) for the bacterial and archaeal portion of the first high-complexity sample. Each error metric was divided by its maximal value to facilitate viewing on the same scale and relative performance comparisons. **(b)** Absolute recall and precision for each profiler on the microbial (filtered) portion of the low-complexity data set across six taxonomic ranks. Red text and asterisk indicate methods for which no predictions at the corresponding taxonomic rank were returned. FS, FOCUS; T-P, Taxy-Pro; MP2.0, MetaPhlAn 2.0; MP, MetaPhyler; CK, Common Kmers; D, DUDes. **(c)** Best scoring profilers using different performance metrics summed over all samples and taxonomic ranks to the genus level. A lower score indicates that a method was more frequently ranked highly for a particular metric. The maximum (worst) score for the UniFrac metric is 38 ($18 + 11 + 9$ profiling submissions for the low, medium and high-complexity data sets, respectively), while the maximum score for all other metrics is 190 (5 taxonomic ranks \times ($18 + 11 + 9$) profiling submissions for the low, medium and high-complexity data sets, respectively).

'filtered' indicates the gold standard without these data. The affected metrics were the abundance-based metrics (L1 norm at the superkingdom level and weighted UniFrac) and precision and recall (at the superkingdom level): all methods correctly detected Bacteria and Archaea, but only MetaPhlAn 2.0 and CLARK detected viruses in the unfiltered samples. Averaging over all methods and samples, L1 norm at the superkingdom level increased from 0.05, for the filtered samples, to 0.29, for the unfiltered samples. Similarly, the UniFrac metric increased from 7.21, for the filtered data sets, to 12.36, for the unfiltered data sets. Thus, the fidelity of abundance estimates decreased notably when viruses and plasmids were present.

Taxonomic profilers versus profiles from taxonomic binning

Using a simple coverage-approximation conversion algorithm, we derived profiles from the taxonomic binning results (Supplementary Note 1 and Supplementary Figs. 42–45). Overall, precision and recall of the taxonomic bidders were comparable to that of the profilers. At the order level, the mean precision over all taxonomic bidders was 0.60 (versus 0.40 for the profilers), and the mean recall was 0.82 (versus 0.86 for the profilers). MEGAN6 and PhyloPythiaS+ had better recall than the profilers at family level, though PhyloPythiaS+ precision was below that of Common Kmers and MetaPhlAn 2.0 as well as the bidder taxator-tk (Supplementary Figs. 42 and 43), and—similarly to the profilers—recall also degraded below family level.

Abundance estimation at higher ranks was more problematic for the bidders, as L1 norm error at the order level was 1.07 when averaged over all samples, whereas for the profilers it was only 0.68. Overall, though, the bidders delivered slightly more accurate abundance estimates, as the binning average UniFrac metric was 7.03, whereas the profiling average was 7.23. These performance differences may be due in part to the gold-standard contigs used by the bidders (except for MEGAN6), though Kraken is also often applied to raw reads, while the profilers used the raw reads.

DISCUSSION

A lack of consensus about benchmarking data and evaluation metrics has complicated metagenomic software comparisons and their interpretation. To tackle this problem, the CAMI challenge engaged 19 teams with a series of benchmarks, providing performance data and guidance for applications, interpretation of results and directions for future work.

Assemblers using a range of *k*-mers clearly outperformed single *k*-mer assemblers (Supplementary Table 1). While the latter reconstructed only low-abundance genomes (with small *k*-mers) or high-abundance genomes (with large *k*-mers), using multiple *k*-mers substantially increased the recovered genome fraction. Two programs also reconstructed high-copy circular elements well, although none detected their circularities. An unsolved challenge for all programs is the assembly of closely related genomes. Notably, poor or failed assembly of these genomes will negatively affect subsequent contig binning and further complicate their study.

All genome bidders performed well when no closely related strains were present. Taxonomic bidders reconstructed taxon bins of acceptable quality down to the family rank (Supplementary Table 1). This leaves a gap in species and genus-level reconstruction—even when taxa are represented by single strains—that

needs to be closed. Notably, taxonomic bidders were more precise when reconstructing genomes than for species or genus bins, indicating that the decreased performance for low ranks is due partly to limitations of the reference taxonomy. A sequence-derived phylogeny might thus represent a more suitable reference framework for "phylogenetic" binning. When comparing the average taxon bidder performance for taxa with similar surroundings in the SILVA and NCBI taxonomies to those with less agreement, we observed significant differences—primarily as a decrease in performance for low-ranking taxa in discrepant surroundings (Supplementary Note 1 and Supplementary Table 8). Thus, the use of SILVA might further improve taxon binning, but the lack of associated genome sequences represents a practical hurdle⁴³. Another challenge for all programs is to deconvolute strain-level diversity. For the typical covariance of read coverage-based genome bidders, it may require many more samples than those analyzed here (up to five) for satisfactory performance.

Despite variable performance, particularly for precision (Supplementary Table 1), most taxonomic profilers had good recall and low error in abundance estimates until family rank. The use of different classification algorithms, reference taxonomies, databases and information sources (for example, marker genes, *k*-mers) probably contributes to observed performance differences. To enable systematic analyses of their individual impacts, developers could provide configurable rather than hard-coded parameter options. Similarly to taxonomic bidders, performance across all metrics dropped substantially below family level. When including plasmids and viruses, all programs gave worse abundance estimates, indicating a need for better analysis of data sets with such content, as plasmids are likely to be present, and viral particles are not always removed by size filtration⁴⁴.

Additional programs can still be submitted and evaluated with the CAMI benchmarking platform. Currently, we are further automating the benchmarking and comparative result visualizations. As sequencing technologies such as long-read sequencing and metagenomics programs continue to evolve rapidly, CAMI will provide further challenges. We invite members of the community to contribute actively to future benchmarking efforts by CAMI.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the [online version of the paper](#).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.C., N.N., C.Q., B.K.H.C., B.D., J.L.F., Z.W., R.E., D.D.K., J.J.C., C.D., C.L., P.P., G.R., D.L., Y.-W.W., S.W.S., C.J., M.S., H.K., P.M., T.L., H.-H.L., Y.-C.L., G.G.Z.S., D.A.C., R.A.E., S.S., V.C.P., B.Y.R., D.K., J.D. and I.G. participated in challenge; P.S.-L., J.A.V., Y.B., T.S.J., L.H.H., S.J.S., N.C.K., E.M.R., T.W., H.-P.K., M.G. and N.S. generated and contributed data; P.H., S.M., J.F., E.D., D.T., M.Z.D., A.S., A.B., A.E.D., T.R. and A.C.M. generated benchmark data sets; P.B. implemented the benchmarking platform; M. Beckstette and P.D.B. provided computational support; D.K., P.H., S.J., J.D., I.G., R.G.-O., C.Q., A.F., F.M., P.B., M.D.B., M. Balvočiūtė and A.G. implemented benchmarking metrics and bioboxes and performed evaluations; D.K., A.S., A.C.M., C.Q., J.D., P.H., T.S.J., D.D.K., Y.-W.W., A.B., A.F., R.C., M.P. and P.B. interpreted results with comments from many authors; A.C.M., A.S. and D.K. wrote the paper with comments from many authors; A.S., T.R. and A.C.M. conceived research with input from many authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Community involvement. We organized public workshops, roundtables, hackathons and a research program around CAMI at the Isaac Newton Institute for Mathematical Sciences (**Supplementary Fig. 46**) to decide on the principles realized in data set generation and challenge design. To determine the most relevant metrics for performance evaluation, a meeting with developers of evaluation software and commonly used binning, profiling and assembly software was organized. Subsequently we created biobox containers implementing a range of commonly used performance metrics, including the ones decided to be most relevant in this meeting (**Supplementary Table 9**). Computational support for challenge participants was provided by the Pittsburgh Supercomputing Center.

Standardization and reproducibility. For performance assessment, we developed several standards: we defined output formats for profiling and binning tools, for which no widely accepted standard existed. Second, we defined standards for submitting the software itself, along with parameter settings and required databases and implemented them in Docker container templates (bioboxes)¹⁰. These enable the standardized and reproducible execution of submitted programs from a particular category. Participants were encouraged to submit results together with their software in a Docker container following the bioboxes standard. In addition to 23 bioboxes submitted by challenge participants, we generated 13 other bioboxes and ran them on the challenge data sets (**Supplementary Table 2**), working with the developers to define the most suitable execution settings, if possible. For several submitted programs, bioboxes using default settings were created to compare performance with default and expert chosen parameter settings. If required, the bioboxes can be rerun on the challenge data sets.

Genome sequencing and assembly. Draft genomes of 310 type strain isolates were generated for the Genomic Encyclopedia of Type Strains at the DOE Joint Genome Institute (JGI) using Illumina standard shotgun libraries and the Illumina HiSeq 2000 platform. All general aspects of library construction and sequencing performed at the JGI can be found at <http://www.jgi.doe.gov>. Raw sequence data were passed through DUK, a filtering program developed at JGI, which removes known Illumina sequencing and library preparation artifacts (L. Mingkun, A. Copeland and J. Han (Department of Energy Joint Genome Institute, personal communication). The genome sequences of isolates from culture collections are available in the JGI genome portal (**Supplementary Table 10**). Additionally, 488 isolates from the root and rhizosphere of *Arabidopsis thaliana* were sequenced⁸. All sequenced environmental genomes were assembled using the A5 assembly pipeline (default parameters, version 20141120)⁴⁵ and are available for download at <https://data.cami-challenge.org/participate>. A quality control of all assembled genomes was performed on the basis of tetranucleotide content analysis and taxonomic analyses (**Supplementary Note 1**), resulting in 689 genomes that were used for the challenge (**Supplementary Table 10**). Furthermore, we generated 1.7 Mbp or 598 novel circular sequences of plasmids, viruses and other circular elements from multiple microbial community samples of rat cecum (**Supplementary Note 1** and **Supplementary Table 11**).

Challenge data sets. We simulated three metagenome data sets of different organismal complexities and sizes by generating 150-bp paired-end reads with an Illumina HighSeq error profile from the genome sequences of 689 newly sequenced bacterial and archaeal isolates and 598 sequences of plasmids, viruses and other circular elements (**Supplementary Note 1**, **Supplementary Tables 3, 6 and 12** and **Supplementary Figs. 47 and 48**). These data sets represent common experimental setups and specifics of microbial communities. They consist of a 15-Gbp single sample data set from a low-complexity community with log normal abundance distribution (40 genomes and 20 circular elements), a 40-Gbp differential log normal abundance data set with two samples of a medium-complexity community (132 genomes and 100 circular elements) and long and short insert sizes, as well as a 75-Gbp time series data set with five samples from a high-complexity community with correlated log normal abundance distributions (596 genomes and 478 circular elements). The benchmark data sets had some notable properties; all included (i) species with strain-level diversity (**Supplementary Fig. 47**) to explore its effect on program performance; (ii) viruses, plasmids and other circular elements, for assessment of their impact on program performances; and (iii) genomes at different evolutionary distances to those in reference databases, to explore the effect of increasing taxonomic distance on taxonomic binning. Gold-standard assemblies, genome bin and taxon bin assignments and taxonomic profiles were generated for every individual metagenome sample and for the pooled samples of each data set.

Challenge organization. The first CAMI challenge benchmarked software for sequence assembly, taxonomic profiling and (taxonomic) binning. To allow developers to familiarize themselves with the data types, biobox containers and in- and output formats, we provided simulated data sets from public data together with a 'standard of truth' before the start of the challenge. Reference data sets of RefSeq, NCBI bacterial genomes, SILVA⁴⁶ and the NCBI taxonomy from 30 April 2014 were prepared for taxonomic binning and profiling tools, to enable performance comparisons for reference-based tools based on the same reference data sets. For future benchmarking of reference-based programs with the challenge data sets, it will be important to use these reference data sets, as the challenge data have subsequently become part of public reference data collections.

The CAMI challenge started on 27 March 2015 (**Supplementary Figs. 46 and 49**). Challenge participants had to register on the website to download the challenge data sets, and 40 teams registered at that time. They could then submit their predictions for all data sets or individual samples thereof. They had the option of providing an executable biobox implementing their software together with specifications of parameter settings and reference databases used. Submissions of assembly results were accepted until 20 May 2015. Subsequently, a gold-standard assembly was provided for all data sets and samples, which was suggested as input for taxonomic binning and profiling. This includes all genomic regions from the genome reference sequences and circular elements covered by at least one read in the pooled metagenome data sets or individual samples (**Supplementary Note 1**). Provision of this assembly gold standard allowed us to decouple the performance analyses of binning and profiling tools from assembly performance. Developers could submit their binning and profiling results until 18 July 2015.

Overall, 215 submissions representing 25 different programs were obtained for the three challenge data sets and samples, from initially 19 external teams and CAMI developers, 16 of which consented to publication (**Supplementary Table 2**). The genome data used to generate the simulated data sets was kept confidential until the end of the challenge and then released⁸. To ensure a more unbiased assessment, we required that challenge participants had no knowledge of the nature of the challenge data sets. Program results displayed in the CAMI portal were given anonymous names in an automated manner (only known to the respective challenge submitter) until a first consensus on performances was reached in the public evaluation workshop. In particular, this was considered relevant for evaluation of taxator-tk and PhyloPythiaS+, which were from the lab of one of the organizers (A.C.M.) but submitted without her involvement.

Evaluation metrics. We briefly outline the metrics used to evaluate the four software categories. All metrics discussed, and several others, are described more in depth in **Supplementary Note 1**.

Assemblies. The assemblies were evaluated with MetaQUAST⁴⁷ using a mapping of assemblies to the genome and circular element sequences of the benchmark data sets (**Supplementary Table 4**). As metrics, we focused on genome fraction and assembly size, the number of unaligned bases and misassemblies. Genome fraction measures the assembled percentage of an individual genome, assembly size denotes the total assembly length in bp (including misassemblies), and the number of misassemblies and unaligned bases are error metrics reflective of the assembly quality. Combined, they provide an indication of the program performance. For instance, although assembly size might be large, a high-quality assembly also requires the number of misassemblies and unaligned bases to be low. To assess how much metagenome data was included in each assembly, we also mapped all reads back to them.

Genome binning. We calculated completeness and purity for every bin relative to the genome with the highest number of base pairs in that bin. We measured the assignment accuracy for the portion of the assigned data by the programs with the ARI. This complements consideration of completeness and purity averaged over genome bins irrespective of their sizes (**Fig. 2c,d**), as large bins contribute more than smaller bins in the evaluation. As not all programs assigned all data to genome bins, the ARI should be interpreted under consideration of the fraction of data assigned (**Fig. 2a**).

Taxonomic binning. As performance metrics, the average purity (precision) and completeness (recall) per taxon bin were calculated for individual ranks under consideration of the taxon assignment. In addition, we determined the overall classification accuracy for each data set, as measured by total assigned sequence length, and misclassification rate for all assignments. While the former two measures allow assessing performance averaged over

bins, where all bins are treated equally, irrespective of their size, the latter are influenced by the taxonomic constitution, with large bins having a proportionally larger influence.

Taxonomic profiling. We determined abundance metrics (L1 norm and weighted UniFrac)⁴⁸ and binary classification measures (recall and precision). The first two assess how well a particular method reconstructs the relative abundances in comparison to the gold standard, with the L1 norm using the sum of differences in abundances (ranges between 0 and 2) and UniFrac using differences weighted by distance in the taxonomic tree (ranges between 0 and 16). The binary classification metrics assess how well a particular method detects the presence or absence of an organism in comparison to the gold standard, irrespective of their abundances. All metrics except the UniFrac metric (which is rank independent) are defined at each taxonomic rank. We also calculated the following summary statistic: for each metric, on each sample, we ranked the profilers by their performance. Each was assigned a score for its ranking (0 for first place among all tools at a particular taxonomic rank for a particular sample, 1 for second place, etc.). These scores were then added over the taxonomic ranks to the genus level and summed over the samples, to give a global performance score.

Data availability. A Life Sciences Reporting Summary for this paper is available. The plasmid assemblies, raw data and metadata have been deposited in the European Nucleotide Archive (ENA) under accession number [PRJEB20380](https://www.ebi.ac.uk/ena/record/PRJEB20380). The challenge and toy data sets including the gold standard, the assembled genomes used to generate the benchmark data sets (**Supplementary Table 10**), NCBI and ARB public reference sequence collections without the benchmark data and the NCBI taxonomy version used for taxonomic binning and profiling are available in GigaDB under data set identifier ([100344](https://gigadb.org/dataset/100344)) and on the CAMI analysis site for download and within the benchmarking platform (<https://data.cami-challenge.org/participate>). Further information on the CAMI challenge, results and scripts is provided at <https://github.com/CAMI-challenge/>. **Supplementary Tables 2 and 9** specify the Docker Hub locations of bioboxes for the evaluated programs and used metrics. Source data for **Figures 1–3** are available online.

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