binny: an automated binning algorithm to recover high-quality genomes from complex metagenomic datasets

Oskar Hickl[1], Pedro Queirós[2], Paul Wilmes[2], Patrick May[1,†] and Anna Heintz-Buschart[3,†,*],

1 Bioinformatics Core, Luxembourg Centre for Systems Biomedicine, University of Luxembourg
2 Systems Ecology, Luxembourg Centre for Systems Biomedicine, University of Luxembourg
3 Biosystems Data Analysis, Swammerdam Institute for Life Sciences, Faculty of Science, University of Amsterdam

*** Correspondence: a.u.s.heintzbuschart@uva.nl**
† Equal contributions.

Abstract

The reconstruction of genomes is a critical step in genome-resolved metagenomics and for multi-omic data integration from microbial communities. Here, we present *binny*, a binning tool that produces high-quality metagenome-assembled genomes (MAG) from both contiguous and highly fragmented genomes. Based on established metrics, *binny* outperforms existing state-of-the-art binning methods and finds unique genomes that could not be detected by other methods. *binny* uses k-mer-composition and coverage by metagenomic reads for iterative, non-linear dimension reduction of genomic signatures as well as subsequent automated contig clustering with cluster assessment using lineage-specific marker gene sets. When compared to five widely used binning algorithms, *binny* provides substantial amounts of uniquely identified MAGs and recovers the most near-complete (>95% pure, >90% complete) and high-quality (>90% pure, >70% complete) genomes from simulated data sets from the Critical Assessment of Metagenome Interpretation (CAMI) initiative, as well as from a real-world benchmark comprised of metagenomes from various environments.

Introduction

High-throughput shotgun sequencing has become the standard to investigate metagenomes [1,2]. Metagenome-assembled genomes (MAGs) allow the linking of the genetic information at species or strain level: In the absence of cultured isolates, MAGs form an important point of reference. Thereby, study-specific MAGs have led to the discovery of previously uncharacterised microbial taxa [3] and deepened insights into microbial physiology and ecology [4,5]. In addition, large system-wide collections, which have been assembled recently, e.g. for the human microbiome [6] and several environmental systems [7], equip researchers with a common resource for short-read annotation. These collections also represent an overview of the pangenomic potential of microbial taxa of interest [8,9]. In addition to facilitating the interpretation of metagenomic data, genome-resolution also provides an anchor for the integration of functional omics [10,11].

However, obtaining high-quality MAGs is still challenging [12]. Most approaches start from assembled contigs, which are then binned by clustering, e.g. expectation-maximization clustering [13,14] or graph-based clustering [15], of k-mer frequency or abundance profiles or both. Therefore, issues with metagenomic assemblies, such as fragmentation of the assembly because of insufficient sequencing depth, repeat elements within genomes, and unresolved ambiguities between closely related genomes, are perpetuated to MAGs. In addition, the features based on which contigs are binned are not generally homogeneous over genomes: for example copy number, and thereby metagenomic coverage, may vary over the replicating genome; certain conserved genomic regions, but also newly acquired genetic material, can deviate in their k-mer frequency from the rest of the genome [12].

In the face of these challenges, the algorithms used to bin assembled metagenomic contigs into congruent groups which form the basis for MAGs, can approximately be evaluated according to a set of criteria [16]. Most importantly, MAGs should be as complete as possible and contain as little contamination as possible.

In metagenomic datasets with defined compositions, such as those provided by the Critical Assessment of Metagenome Interpretation (CAMI) initiative [17–19], the evaluation can be achieved by comparison with the reference genomes. For yet un-sequenced genomes, completeness and contamination can be assessed based on the presence and redundancy of genes that are expected to be present as single copies in many [20] or all [21] bacteria or archaea [22], or in specific lineages [23]. Contiguity and GC-skew provide further measures for highly complete genomes [12]. For reporting and storing MAGs in public repositories, the MIMAG standard has been proposed [24]. In addition to completeness and contamination based on protein-coding genes, this standard also takes into account the presence of tRNAs and rRNAs. The latter present particular challenges for assembly and binning methods alike [12]. Nevertheless, the recruitment of rRNA genes to MAGs would improve the association with existing MAG collections [6, 25] and rRNA-gene based databases [26], which are widely used for microbial ecology surveys. In addition to binning tools, methods that refine MAGs by complementing results from multiple binning methods have been developed [27, 28]. These generally improve the overall yield and quality of MAGs [29]. Finally, manual refinement is still recommended and is supported by multiple tools [12, 30–33].

Here, we present *binny*, an automated binning method that was developed based on a semi-supervised binning strategy [10,34]. *binny* is implemented as a reproducible Python-based workflow using Snakemake [35]. *binny* is based on iterative clustering of dimension-reduced k-mer and abundance profiles of metagenomic contigs. It evaluates clusters based on the presence of lineage-specific single copy marker genes [23]. We benchmarked *binny* against six CAMI [17, 18] data sets and compared the results to the most popular binning methods MetaBAT2 [15], MaxBin2 [14], CONCOCT [13], and the recently developed VAMB [36]. We evaluated the contribution of *binny* to automatic MAG refinement using MetaWRAP [27] and DAS Tool [28]. Finally, we evaluated the MAGs returned by all approaches from real-world metagenomic datasets from a wide range of ecosystems. We report that *binny* outperforms existing methods in terms of completeness and purity and improves combined refinement results. *binny* also returned most high-quality and near-complete MAGs from both highly fragmented and more contiguous metagenomes over a range of microbial ecosystems.

Materials and Methods

binny workflow

binny is implemented as a Snakemake [35] workflow (Figure 1). At the centre of the workflow is the binning algorithm written in Python, which uses iterative, nonlinear dimension reduction of metagenomic read coverage depth and *k*-mer signatures and subsequent automated contig clustering with cluster assessment by lineage-specific marker gene sets. Preparatory processing steps include calculation of average depth of coverage, gene calling using Prokka [37], masking of rRNA gene and CRISPR regions on input contigs, and identifying CheckM [23] marker genes using Mantis [38].

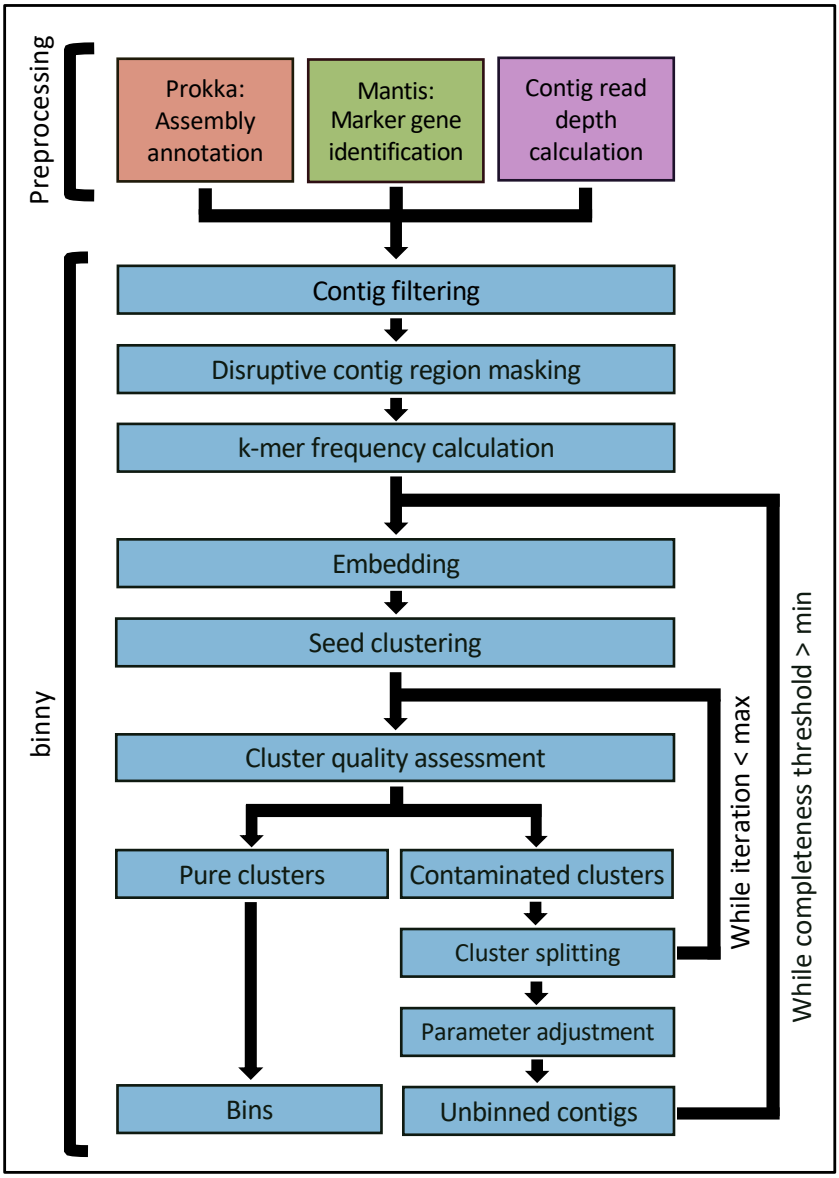

Figure 1. *binny* workflow. Overview of the Snakemake pipeline and of *binny*'s binning method. Preprocessing includes assembly annotation using Prokka, CheckM marker gene detection using Mantis, and (optional) average contig read coverage calculation. *binny* filters out contigs shorter than the specified value, masks potentially disruptive contig regions before calculating k-mer frequencies for the chosen k-mer size(s). In its main routine, *binny* iteratively embeds the contig data into two dimensional space, forms clusters, assesses them with marker genes, and extracts high quality clusters as MAGs.

binny operates in an iterative manner after processing of the annotated marker gene sets: Each iteration consists of non-linear dimension reduction on the selected features (depths of coverage and *k*-mer frequencies)

and clustering of the so far unbinned contigs based on the resulting two-dimensional coordinates. Clusters are selected, if the contained marker gene sets indicate purity and completeness above defined thresholds. A new iteration is started on left-over un-binned contigs with dynamically adjusted parameters. Finally, clusters above the thresholds are output as MAGs.

Marker gene set processing *binny* generates a directed graph database of the CheckM [23] taxon-specific marker sets annotated per contig in NetworkX [39]. This allows for fast access to the hierarchical (lineage-based) information. Some marker sets are omitted, as they are very small and/or led to imprecise assessments in testing (Supplementary Table 1).

Filtering of short sequences *binny* filters assemblies based on an NX value (default 95), to retain only those largest contigs that make up X% of the assembly. The size selection can be further modified by setting minimum size ranges for contigs which do not contain marker genes (default 500-1000 bp) and those which contain them (default 250-400 bp). This aims to maintain the maximum amount of information from an assembly, because only contigs which have a low information content are omitted.

Masking of disruptive sequence regions Repetitive regions on a sequence could possibly skew the k-mer frequency significantly and, thus, adversely affect the binning process. To avoid this and still keep sequences intact, *binny* by default masks sequence elements/regions such as rRNAs and CRISPR regions, using the annotations from barrnap [37] and *minced* [40], respectively, in Prokka, so that they are ignored during the k-mer frequency calculation. CRISPR regions contain foreign genetic elements, which have k-mer frequencies that can deviate substantially from the rest of the genome, while rRNAs have highly conserved sequences whose k-mer profiles do not resemble the rest of a given genome.

Single contig genome recovery Because (near-)complete genomes represented by single contigs might not be distinguishable from noise or be clustered together with highly similar contigs of other genomes during the clustering step, their separation and subsequent recognition as pure and complete is hindered. Therefore, contigs with at least 40 different markers are extracted first and assessed. If they are at least 90% pure and 92.5% complete, they are kept as single-contig MAGs and do not enter the iterative binning procedure.

Binning features *binny* uses two features of the contigs for dimensionality reduction and clustering: the k-mer frequencies (default $k = 2$, 3, and 4) and average depth of read coverage (raw read counts of one or more samples) centered log ratio transformed. If available, multiple sources of depth of coverage information can be included in form of bam files or a file with tab-separated average contig depth of coverage values per sample.

Dimensionality reduction To reduce the dimensionality of all features to two the Fast Fourier Transform-accelerated Interpolation-based t-distributed Stochastic Neighbor Embedding (FIt-SNE) implementation of openTSNE [41] is used. To decrease the computation time of the dimensionality reduction, Principal Component Analysis (PCA) is used beforehand to lower the dimensionality of the initial feature matrix to either as many dimensions needed to explain 75% of the variation or to a maximum of 75 dimensions. To improve the embedding quality, especially with large datasets, multiple strategies are used: i) A multi-scale kernel with perplexity ranges from 10-20 and 100-130 starting with 10 and 100, where each iteration the former is increased by 2 and the latter by 5, are used instead of a Gaussian model to balance out local and global structure, as described by Kobak and Berens [42]. ii) An early exaggeration of *EX* for the number of unbinned contigs *NUC*:

$$\mathrm{EX} = min\{4, max\{100, \mathrm{NUC} \times 2.5 \times 10^{-4}\}\} \tag{1}$$

with a learning rate *LR_EX* for the early exaggeration phase:

$$\mathrm{LR_EX} = max\{2, \frac{\mathrm{NUC}}{\mathrm{EX}}\} \tag{2}$$

and a learning rate *LR*:

$$\mathrm{LR} = max\{200, min\{64 \times 10^3, \mathrm{NUC} \times 0.1\}\} \tag{3}$$

for the main phase are used. These values were chosen to achieve high quality embeddings of data sets of varying sizes [43, 44]. Additionally, the number of iterations to run early and main phase optimizations

are based on the difference in Kullback-Leibler divergence (*KLD*) *KLD_DIFF*. KLD is measured every 250 iterations, so that the optimization is ended, if:

$$\text{KLD_DIFF} < \text{KLD} \times 0.01 \tag{4}$$

as done similarly in Belkina et al. [44]. iii) To avoid the impreciseness of Euclidean distance measures in high dimensional space, Manhattan distance was chosen instead [45]. Default values were kept for all other openTSNE parameters.

Iterative clustering *binny* uses hierarchical density-based spatial clustering of applications with noise (HDBSCAN) [46] on the generated two dimensional embedding, in iterations. *binny* will cluster the created embedding n times, where n is the number of values for HDBSCAN's minimum number of samples parameter (default 1,2,4,6,8, n=5), each time extracting MAGs meeting the quality thresholds and continuing with the embedding containing only the leftover contigs.

Other default clustering parameters are: The minimum cluster size is calculated with ln(*n contigs*), the cluster selection epsilon to merge micro-clusters is changed each *binny* iteration, cycling from 0.5 to 0.0 in 0.125 steps, and the distance metric used is Manhattan.

For each cluster, completeness and purity are assessed (see below). If a cluster passes the completeness threshold (by default starting with 90% and then decreasing to a minimum of 70%) and has a purity above 90%, it is kept as a MAG. Otherwise, *binny* will attempt to split that contig cluster iteratively using HDBSCAN a set maximum amount of times (see above) but adding the raw depth(s) of coverage as additional dimension(s). Within each of these clustering rounds, the clusters below the quality threshold are split again using HDBSCAN until no new clusters are identified and/or the maximum number of iterations is reached (default 2). To prevent the selection of low purity clusters, the purity threshold is increased continuously to a maximum of 95% at completeness 70% or lower (99%, if the chosen marker set is Bacteria or Archaea).

Cluster assessment using marker gene sets Clusters are assessed by calculating the purity and completeness based on the CheckM marker grouping approach, where marker genes known to be co-located in genomes of a lineage are collapsed into marker sets [23]. *binny* calculates MAG quality as in Parks et al. equation 1 and 2, respectively [23], except that instead of contamination purity is calculated: Let P be the purity for a set of collocated marker sets *MSS*, *MS* a marker set in *MSS*, g a single copy marker gene in *MS*, and C the counts of g in a MAG:

$$\mathrm{P_{MSS}} = \frac{\sum_{\mathrm{M} \in \mathrm{MS}} \frac{\sum_{\mathrm{g} \in \mathrm{M}} \frac{1}{C_g}}{|M|}}{|\mathrm{MS}|} \tag{5}$$

The taxonomic level and identity of the marker set is chosen dynamically: Assessment starts with completeness and purity of the domain-level marker sets and traverses the lineage down one taxonomic level at a time, at each level calculating completeness and purity for each taxon of the lineage at that level. To combine the power of the domain level marker sets to give a general quality assessment and the specificity of lower level marker sets, the mean of purity and contamination for sub-domain level markers sets with its respective domain level set is used.

If the current marker set has a equal or higher completeness than the previously best fitting marker set, it is set as the new reference. The reason being that the marker set with the highest completeness is least likely to be matching by chance and fidelity scales with marker set size.

Iterative embedding *binny* creates embeddings of the un-binned contigs of each clustering and runs subsequent clustering iterations, for as long as it finds new MAGs that satisfy the purity and completeness thresholds. By default, the completeness threshold is decreased by 10% in every clustering iteration where the median number of MAGs found per sub-clustering iteration is below 1, down to the minimum completeness threshold (70% completeness). Once the minimum completeness threshold is reached, *binny* runs final rounds with different minimum contig sizes starting at 2000bp, decreasing by 500 each round, until 0 or the minimum size set by the user is reached.

Contig depth calculation If not provided explicitly, the average depth of coverage calculation can be performed directly from given BAM files within the Snakemake workflow using BEDTools [47] *genomeCoverageBed* and an in-house Perl script.

Coding sequence, rRNA gene, and CRISPR prediction by Prokka A modified Prokka [37] executable is run with `--metagenome`, to retrieve open reading frame (ORF) predictions from Prodigal [48], rRNA gene predictions from barrnap [37] and CRISPR region predictions from *minced* [40]. The modification improves speed by omitting the creation of a GenBank output and by the paralellisation of the Prodigal ORF prediction step. Additionally, it allows the output of partial coding sequences without start- and/or stop-codons, which are frequently encountered in fragmented assemblies. No functional annotations of the called coding sequences are performed. The GFF output of Prokka is used in the subsequent steps.

Marker gene set annotation Taxon-specific marker gene sets are acquired from CheckM [23] `https://data.ace.uq.edu.au/public/CheckM_databases/` upon installation of *binny*, hidden Markov profile models (HMM) of marker genes not found in `taxon_marker_sets.tsv` are removed, and `checkm.hmm` is split into Pfam [49] and Tigrfam [50] parts. Mantis [38] is used to annotate coding sequences using the two HMM sets. Because both resources are of different scope and quality, consensus generation weights of 1.0 and 0.5 are used for Pfam and Tigrfam models, respectively. Mantis' heuristic search algorithm is used for hit processing, the e-value threshold is set to 1×10^{-3}, and the `--no_taxonomy` flag is set.

Selection of default values for minimum contig size To determine an optimal minimum contig size value, *binny* was run with five pairs of fixed values for contig size and size of contigs with CheckM markers, ranging from 0 to 2000 in 500 bp steps on 54 simulated samples (see section Synthetic benchmark data below) from six data sets of the CAMI initiative [17, 19]. The contig number limit was set to 10×1^7 to effectively remove any limitation. Additionally, a dynamic setting based on the N95 for the assembly, with a minimum contig size range from 500-1000 bp and a minimum size of contigs with CheckM markers range from 250-400 bp, and a global contig number limit of 3.5×10^5 was tested. The dynamic setting was chosen as default parameter, as it produced a high number of MAGs with a purity > 95% and a completeness > 90%, with other key metrics being similar between all parameter sets all the while using a realistic threshold for the contig number (Supplementary Figure 1).

Parameter customization To optimize for their use case, a user can choose to change the sizes and number of k-mers used, the NX value and/or minimum contig length to filter the assembly, as well as the minimum completeness and purity thresholds for bins. The user may choose not to mask potentially disruptive regions and can control the clustering process by adjusting several HDBSCAN parameters. Additionally, it is possible to chose between internal calculation of the average contig read depth or supply of a depth value file.

Requirements/dependencies *binny* is implemented as a Snakemake pipeline and an installation script is provided, which takes care of the installation of all necessary dependencies and required databases.

Benchmarking

Synthetic benchmark data Binning performance was evaluated using data sets from the CAMI initiative [17, 19]. To benchmark against data of varying complexity, five short-read data sets with a total of 49 samples were chosen from the 2nd CAMI Toy Human Microbiome Project Dataset (`https://data.cami-challenge.org/participate`). Additionally, to test against a very large data set, the five sample Toy Test Dataset High Complexity from the first CAMI challenge (`https://openstack.cebitec.uni-bielefeld.de:8080/swift/v1/CAMI_I_TOY_HIGH`) was used.

To test the performance on co-assembled data, the pooled assemblies of each of the six CAMI datasets and the respective number of sample read files for each data set, provided by the CAMI challenges, were used. Contig read depth per sample was calculated using *binny* and provided to all binning methods unless stated otherwise. Read files were de-interleaved (`https://gist.github.com/nathanhaigh/3521724#file-deinterleave_fastq-sh`) and mapped against the contigs using `bwa-mem` [51].

Real world benchmark data To assess the binning performance in different real-world scenarios with a variety of metagenome sizes, complexities and qualities, 105 metagenomes used in the MetaBAT2 publication [15] for benchmarking were chosen based on the availability of preprocessed read data at the Joint Genome Institute (JGI). The newest available assembly for the metagenomes and the respective preprocessed reads were retrieved from JGI (`https://jgi.doe.gov/`). The read data was processed in the same way as the CAMI data. For a full list with all sample information see Supplementary Table 2.

Other binning and refinement methods The performance of *binny* was compared to four other state-of-the-art binning methods, and to two binning refinement tools. They were all run using the default settings, unless specified otherwise: MaxBin2 (2.2.7) [14] was run by providing the contig read depth files using the `-abund` option, and with the `-verbose` option. MetaBAT2 (2.2.15) [15] was provided the contig read depth files using the `-a` option, and the options `-cvExt`, `--saveCls`, as well as `-v`. CONCOCT (1.1.0) [13] was run following the 'Basic Usage' section in the documentation (`https://concoct.readthedocs.io/en/latest/usage.html`). VAMB (3.0.2) [36] was run with the default parameters and using the Snakemake pipeline as described in the documentation at `https://github.com/RasmussenLab/vamb/blob/master/README.md`. Because VAMB is designed to achieve optimal performance through the combination of the data of multiple samples, the samples from each of the six CAMI data sets were concatenated and run together, as described by the authors (README sections `Recommended workflow` and `Snakemake workflow`). For the real-world metagenomes, samples sharing a JGI GOLD Study ID were run together. As VAMB could not be successfully run on some of the real-world samples using default values, or when trying with lower values of `-m` and `--minfasta`, the number of MAGs recovered was counted as zero for these samples. For a list of these samples see Supplementary Table 3. DAS Tool (1.1.2) [28] was run using Diamond [52] as a search engine on the unfiltered binning method outputs. MetaWRAP (1.2.2) [27] was set to output only contigs with less than 10% contamination and at least 70% completeness and was also provided the unfiltered binning method outputs. Both refinement tools, DAS Tool and MetaWRAP, were run: i) per sample using the data of all five binning methods and ii) all binning methods except *binny*, to asses how many MAGs *binny* contributes in an ensemble approach.

MAG quality standards To match real world workflows, all binning outputs were assessed using CheckM (1.0.12) [23] and filtered to contain only MAGs with a purity > 90% and a completeness > 70%. The latter threshold was set in accordance with the CheckM publication, which suggests that CheckM results are reliable at completeness equal or larger than 70%. MAGs above these thresholds are subsequently called "high quality" (HQ) MAGs. MAGs with a purity > 95% and a completeness > 90% are called "near complete" (NC) MAGs, as defined by Bowers et al. [24].

Additionally, the minimum information about a metagenome-assembled genome (MIMAG) definition of high-quality MAGs was employed, requiring at least 18 unique tRNAs and three unique rRNAs to be present in the MAG in addition to a purity of > 95% and a completeness of > 90% [24].

Besides the recall in terms of bp of the assembly recovered, the read recruitment of MAGs was assessed. All reads mapping as primary mappings to contigs of a MAG were counted per sample and divided by the total read count (forward + reverse) using pysam (`https://github.com/pysam-developers/pysam`).

Assessment of benchmark results Results for the CAMI benchmark were processed using AMBER (2.0.3) [53], a genome reconstruction evaluation tool, with the following parameters, `-x "50,70,90"` and `-k "circular element"`.

To evaluate a MAG, AMBER selects the gold standard genome with the highest share of bps in that MAG as the reference. In contrast to CheckM, where purity and completeness refer to the amount of marker genes present or duplicated, within AMBER and using an available a gold standard, purity and completeness refer to the amount of bp of the reference genome recovered for completeness, and the share of bp of a given MAG with a given reference genome, respectively. Additionally, to assess one or multiple data sets taken together, AMBER defines overall completeness as '*Sum of base pairs coming from the most abundant genome in each predicted genome bin divided by the sum of base pairs in all predicted bins. ...*', and overall purity as '*Sum of base pairs coming from the most abundant genome in each predicted genome MAG divided by the sum of base pairs in all predicted bins. ...*'.

Purity and completeness values are reported as the per data set average, unless specified otherwise. For the real-world benchmarks the average proportion of bp recovered or the number of MAGs recovered is reported together with the standard error of the mean (SEM). Another metric used is the adjusted Rand index (ARI), which is a commonly used metric to measure how similar two datasets are by also applying a multiple testing correcting. Trying to make the comparisons between different binning methods as realistic, fair and transparent as possible, we report all metrics derived from the CheckM-filtered binning results, unless specified otherwise.

To assess the intersections of MAGs formed by the different binning methods on multi-sample datasets, genomes were counted separately for each sample. To this end, the gold standard genome name was concatenated with the sample id to yield unique identifiers for each genome in each sample. All other figures

were created using the Python libraries *matplotlib* [54] and *Seaborn* [55], as well as *UpSetPlot* [56], setting the minimum intersection size to be shown to five, for the UpSet plots. Remaining data analyses were performed and table outputs created using the Python *NumPy* and *pandas* libraries.

Results

Performance on synthetic data sets

To assess *binny*'s performance, six datasets from the Critical Assessment of Metagenome Interpretation (CAMI) initiative were chosen: the high complexity toy dataset of the first CAMI iteration to investigate how *binny* performs on very large data sets, and the five toy human microbiome data sets of the second CAMI iteration to evaluate the performance on a wide range of microbiome sizes and complexities.

Over all six data sets (54 samples), *binny* recovered 36.7% (SEM 2.9%) of the reference genome lengths in the samples as NC MAGs (n=1617) and 44.3% (SEM 3.0%) as HQ MAGs (n=2058), with median recall values of 28.9% and 38.3%, respectively (Figure 2, Supplementary Table 4). In total, 45.6% of the reference genomes where recovered at a purity of 98.4% with an ARI of 0.977 (Supplementary Figure 2, Supplementary Table 5).

For the high complexity data set, *binny* recovered 30.7% of the total reference genomes with a purity of 97.8% , and an ARI of 0.971. In total 358 NC and 517 HQ MAGs were recovered (Supplementary Figure 3, Supplementary Table 6).

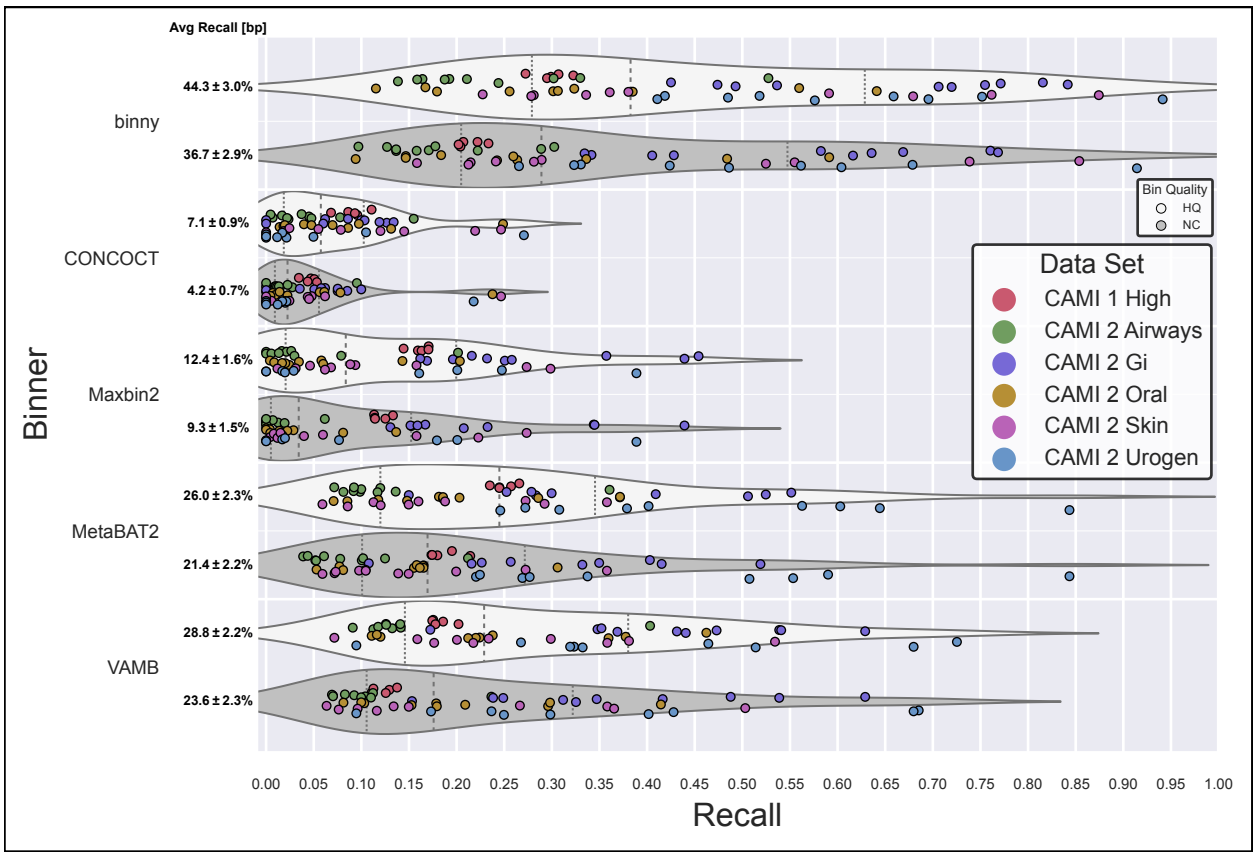

Figure 2. Performance of binning methods on CAMI data sets. Recall of bp assembled sequences as HQ (high-quality) and NC (near-complete) MAGs per binning method per sample, for the six CAMI data sets. The average recall is shown with the standard errors of the mean.

The lowest recall was observed for the CAMI 2 Airways data set with 25.1%, a purity of 98,2% , and an ARI of 0.977 (Supplementary Figure 4), while the highest recall of 67.4%, with a purity of 98,7% , and an ARI of 0.978 (Supplementary Figure 5) was reached with the CAMI 2 Gastrointestinal (GI) data set. For the other three data sets, *binny* achieved the following respective recall, purity and ARI numbers: 62.7%, 98,0%, and 0.971 (CAMI 2 Urogenital); 48.9%, 98,8%, and 0.984 (CAMI 2 Skin); and 33.2%, 98,4%, and 0.980 (CAMI 2 Oral) (Supplementary Figure 6-8, Supplementary Table 6; for detailed metrics for MAGs and samples see Supplementary Tables 7 and 8, respectively.).

Average read recruitment from the CAMI data of the *binny* output was 73.2%. The highest recruitment was achieved for the GI data set sample 5 with 99.3%, while the lowest was observed for the skin dataset

sample 19 (43.9%). Notably, a substantial proportion of the reads recruited were mapped to single contig MAGs for the CAMI 2 datasets (on average 60.6%), while for the CAMI 1 datasets, only about a fifth of the reads recruited by binned contigs, mapped to single contig MAGs (Table 1, Supplementary Tables 9, 10).

Running *binny* with multiple depth files

When assessing the performance on co-assembled datasets, *binny* had a recall of 54.0% over the CAMI datasets with a purity of 98.4%. In total 1062 NC MAGs were produced, 424 of which contained five or more contigs (Supplementary Figure 9-11). The highest recall was achieved for the CAMI 2 Gastrointestinal co-assembly with 75.5% and a purity of 99.0%, while the worst performance was observed for the CAMI 2 Airways dataset with a recall of 32.3% and a purity of 98.2% (Supplementary Table 11,12).

To test to which degree *binny* makes use of the information from the multiple read depth files per co-assembly, *binny* was additionally run with only one depth file per co-assembly. *binny* using all available depth files had a 9.0% higher recall at a slightly higher purity, leading to a recovery of 24.4% more NC MAGs (208) in total and 92.7% more NC MAGs (204) of contig sizes larger than 5 (Supplementary Figure 9-11, Supplementary Table 11,12).

Effect of masking potentially disruptive sequence regions

To test the effect of masking potentially disruptive sequences, we also ran *binny* on the 54 CAMI samples without the masking procedure. The unmasked, unfiltered run did not differ substantially from the unfiltered one with the default settings regarding assembly recall and purity, the latter of which was lower by 1.1%. Additionally, the ARI was reduced 0.04 and five fewer NC MAGs were recovered (Supplementary Table 13). The amount of MAGs recovered matching the MIMAG standard was reduced by 3.7% from 1201 to 1158.

Run time

For all experiments, *binny* was run on compute nodes equipped with AMD Epyc ROME 7H12 CPUs, and for the run-time benchmark 32 cores and 56 GB of RAM were used. For the CAMI samples, the complete *binny* pipeline took on average 170 minutes to run, with a max of 503 minutes for sample five of the CAMI 1 high complexity data set. The Prokka annotations took on average 31 minutes, the Mantis annotations on average 16 minutes, and *binny* on average 121 minutes (Supplementary Table 14).

binny outperformed state-of-the-art binning methods on synthetic data sets

Over all six CAMI datasets *binny* recovered per sample the highest portion of the assembly (bps) as HQ (44.3%) or NC (36.7%) MAGs, followed by VAMB (28.0%, 23.4%) and MetaBAT2 (25.4%, 21.2%). Additionally, *binny* showed the highest median MAG counts with 44.1%, 81.5% more NC and 31.3%, 80.0% more HQ MAGs than VAMB and MetaBAT2, respectively (Figure 2, Supplementary Table 4).

binny was the only binning method that resulted in high purity (97.7%) and high ARI (0.971) output over all datasets without additional CheckM filtering. Using CheckM filtering, *binny*'s purity and ARI were increased by 0.7% and 0.006, respectively, while the assembly recall was decreased by 2.4% (Supplementary Figure 2 b,c, Supplementary Table 5). The second best binning tool, VAMB, had a purity of 75.3% natively and an ARI of 0.675. After CheckM filtering, the purity and ARI of VAMB was the highest among binning methods (99.5% purity and an ARI of 0.994, respectively), but at the same time the recall was reduced from 56.7% to 28.5% (Supplementary Figure 2 b,c, Supplementary Table 5). For detailed metrics on the MAGs and samples see Supplementary Tables 7 and 8, respectively.

binny also outperformed the other binning methods on each of the individual data sets (Figure 2, Supplementary Figure 3-8, Supplementary Table 6).

Many of the CAMI samples contain larger amounts of single-contig or almost contiguous genomes than are commonly observed in real-world samples. To evaluate *binny*'s performance without those, we considered the subset of genomes which consisted of more than five contigs. Here, *binny* also produced substantially more NC (40.7%) and HQ (36.0%) MAGs than VAMB overall, as well as the most unique MAGs found between the best performing binning methods *binny*, VAMB and MetaBAT2. *binny* recovered the largest amount of NC MAGs for all CAMI 2 datasets and recovered substantially more genomes from the CAMI 1 high complexity data set than any of the other binning methods (Supplementary Figure 9). Looking at the assembly recall, *binny* again showed the best performance for each data set (Supplementary Figure 10).

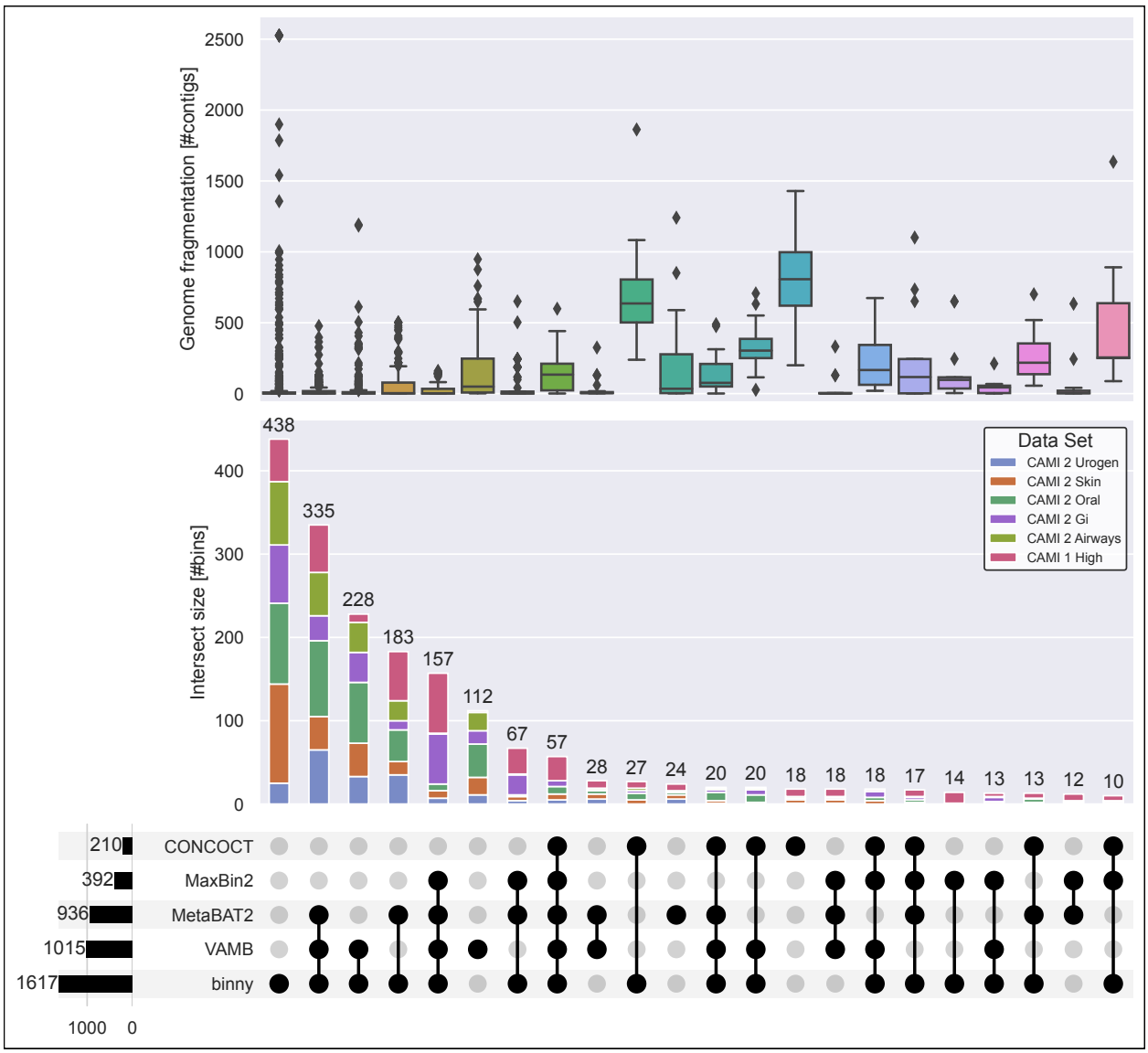

Figure 3. Intersections of recovered CAMI NC MAGs and reference genome fragmentation grade. Intersections of NC (near-complete) MAGs of five CheckM-filtered binning methods for 54 samples from six CAMI data sets. Upper panel: Reference genome fragmentation in number of contigs. Middle panel: Intersection size in number of NC bins with proportions of bins stemming from the six CAMI data sets. Lower panel: Number of bins per binning method on the left, intersections > 4 in the centre.

Additionally, *binny* surpassed the second and third best performing binning methods of the single sample assembly benchmark, VAMB and MetaBAT2, on the co-assembly versions of the six data sets. It recovered 86.0% more NC MAGs of any fragmentation grade and 63.7% more NC MAGs of genomes consisting of more than five contigs than the second best performer, MetaBAT2 (Supplementary Figure 11-13, Supplementary Table 11,12).

Lastly, we assessed the amount of MAGs meeting the MIMAG high quality draft standard. *binny* recovered the most MAGs of that quality for each CAMI data set, recovering in total 50.3% more than the second best method with 1201 (Table 1).

binny recovered most unique MAGs as well as many MAGs also found by other binning methods

To evaluate the performance of different binning tools, it is also of interest to see how much unique information is recovered by each individual binning method. *binny* yielded substantially more unique NC MAGs (438)

Table 1. MIMAG High Quality Draft MAGs recovered by binning methods. Rows represent values for the six CAMI data sets, the sum over all of them, and the number for the real-world benchmark data (IMG). Bold values show the highest count per data set, underlined values the second highest.

	High	Airways	GI	Oral	Skin	Urogen	Total	IMG
binny	**182**	**185**	**200**	**252**	**226**	**156**	**1201**	**570**
CONCOCT	17	10	19	37	18	6	107	142
MaxBin2	85	5	79	20	26	16	231	<u>422</u>
MetaBAT2	<u>144</u>	81	100	134	85	111	655	417
VAMB	107	<u>121</u>	<u>138</u>	<u>197</u>	<u>123</u>	<u>113</u>	<u>799</u>	406

than the other methods for the CAMI data sets, followed by VAMB (112), MetaBAT2 (24), CONCOCT (18), and MaxBin2 (2). Additionally, the two largest sets of MAGs shared by two binning methods are both *binny* sharing MAGs with VAMB (228) or MetaBAT2 (183), respectively (Figure 3). For the HQ genomes, similar results were observed: *binny* recovered the most unique MAGs and was present in almost all of the intersections with the largest numbers of genomes (Supplementary Figure 14).

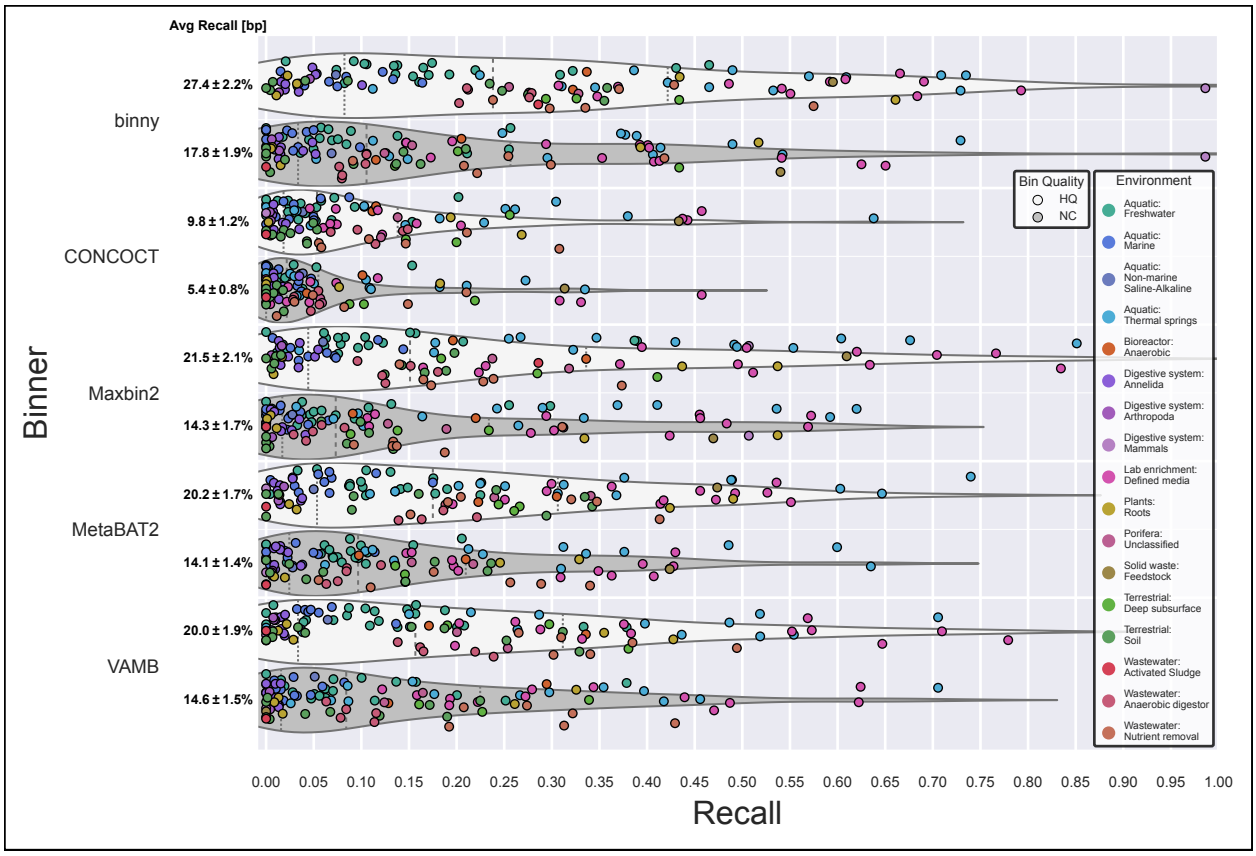

Figure 4. Performance of binning methods on real-world data sets from various environments. Assembly recovery as HQ (high-quality) and NC (near-complete) MAGs per binning method per sample from 105 real-world samples. The average recall (% bp) is shown with the standard error of the mean.

binny produced high quality MAGs from contiguous as well as highly fragmented genomes

Next, we assessed the ability of different binning methods to recover genomes of different fragmentation grades. *binny* recovered more highly fragmented genomes (defined here as genomes with more than 500

contigs) than MaxBin2, MetaBAT2 and VAMB. Only CONCOCT, which on other metrics performed poorly, recovered more highly fragmented genomes than *binny*, while both methods shared the recovery large portion of fragmented genomes (Figure 3).

binny recovered a larger number of high-quality MAGs than other binning methods for real-world assemblies from different environments.

When benchmarking binning tools with real-world data from a wide variety of environments, *binny* recovered on average the largest amount of the assembly (bp) as NC (17.8%) and HQ (27.4%) MAGs, producing 8.4% more NC and 23.6%, more HQ MAGs than the binning tool with the second highest number, MetaBAT2. While MetaBAT2 had a comparable median assembly recovery in NC MAGs (10.5% vs 9.7%), *binny* showed a substantially higher median value for HQ MAGs (23.8% vs 17.5%). As in the CAMI benchmarks, CONCOCT showed the lowest recall for both NC and HQ MAGs, while MaxBin2 performed comparatively better with this data than in the CAMI benchmark (Figure 4, Supplementary Table 15, 16). When counting the recovered MIMAG high quality genomes, *binny* produced 570 MAGs, 35.1% more than the second best binning method, MaxBin2 (422; Table 3).

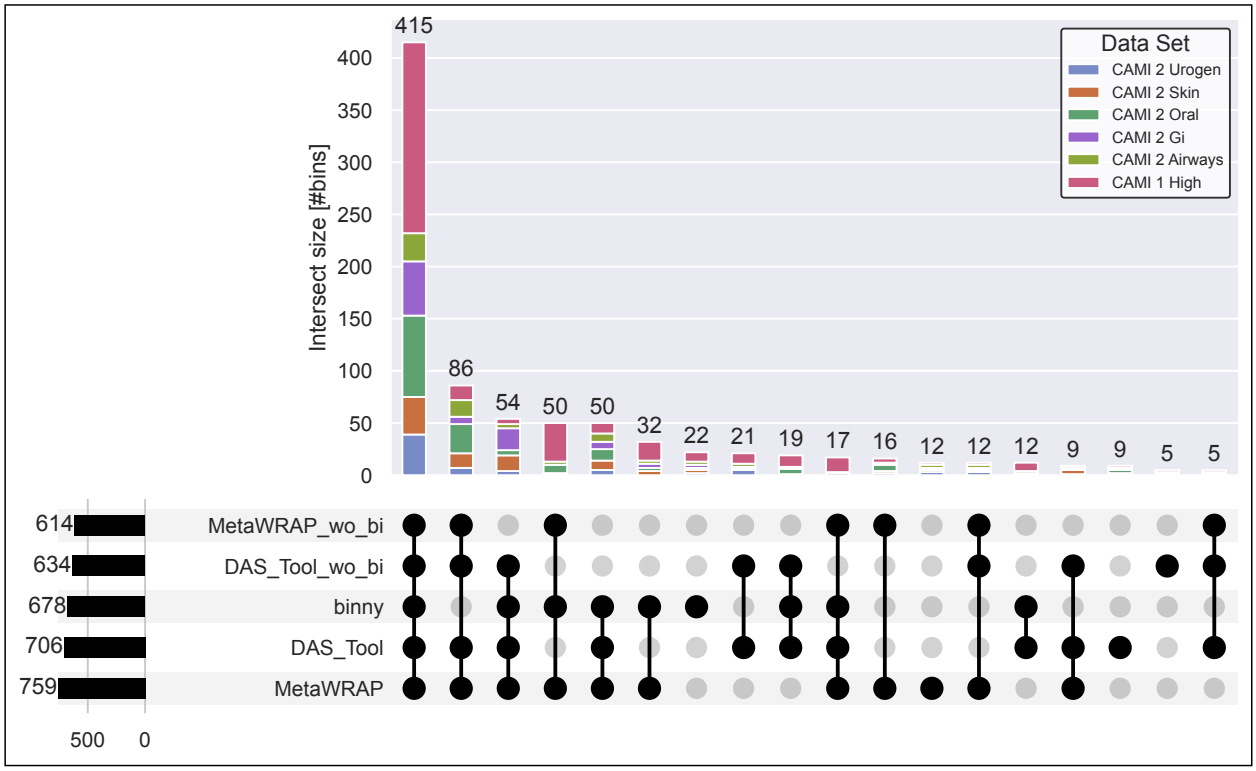

Figure 5. Intersections of recovered CAMI NC MAGs from bin refinement methods. Intersections of NC (near-complete) MAGs from genomes consisting of more than 5 contigs by *binny*, DAS Tool and MetaWrap for 54 samples from six CAMI data sets. Binning method output used by the refinement methods: CONCOCT, *binny*, MaxBin2, MetaBAT2, and VAMB or all but *binny* (_wo_bi). Upper panel: Intersection size in number of NC bins with proportions of bins stemming from the six CAMI data sets. Lower panel: Number of bins per binning method on the left, intersections > 4 in the centre.

binny improved ensemble binning/refinement approaches

To test if *binny* is able to improve refinements in combination with other binning methods, we ran the two most popular automatic refinement tools, DAS Tool and MetaWRAP, on the 54 samples of the six CAMI data sets, combining all other tested binning methods either with or without *binny*.

When *binny* was excluded, a 9.2% and 10.9% lower recall was observed for DAS Tool (42.2%) and MetaWRAP (38.9%), respectively (Supplementary Figure 15 b, c). *binny* on its own recovered more NC MAGs than both refinement tools without *binny* input. When including *binny*, MetaWRAP was able to

recover 9.6% more NC MAGs (1772) than *binny* on its own, while DAS Tool produced 4.8% more NC MAGs (1695) (Supplementary Figure 15 a, Supplementary Figure 16, Supplementary Table 17, 18). Including only MAGs with more than five contigs, the DAS Tool and MetaWRAP without *binny* still performed worse than *binny* on its own (6.9% and 10.4% fewer NC MAGs, respectively). The runs including all five binning methods showed the highest performance overall, with MetaWRAP recovering the most MAGs (Figure 5, Supplementary Figure 17). While MetaWRAP produced almost no heavily contaminated MAGs, DAS Tool returned large numbers of MAGs with very low purity, despite showing over the entire CAMI benchmark data high purity (Supplementary Figure 15 d, Supplementary Table 17, 18)).

Discussion

binny implements a fully automated binning tool, recovering unique information in form of high quality, pure MAGs. It combines k-mer-composition, read coverage, and lineage-specific marker gene sets for iterative, non-linear dimension reduction of genomic signatures and subsequent automated contig clustering with cluster assessment. The low dimensional embedding strategy to reduce large amounts of features has been used before for binning to aid the clustering of contigs [34, 57]. Clustering algorithms perform better in fewer dimensions, because distance information becomes increasingly imprecise at higher dimensions and the chance of random correlation between features rises [58].

While there are already binning methods available that, to some extent, make use of marker genes [14, 59] and also lower dimensional embedding of contig features [59], *binny* uses a new and unique iterative embedding and clustering strategy. Importantly, it assesses clusters of contigs during its iterations, recognizing when further splitting of clusters is necessary. As this lowers the complexity of each clustering task, *binny* recovers genomes that might not be separable with only a single embedding or clustering attempt. This seems to work particularly well for large, complex communities as shown for the five CAMI 1 High complexity samples.

In combination with the ability to incorporate also short informative contigs, *binny* is able to deal with highly fragmented genomes as shown for the CAMI samples, something which, of the tested binning methods, only CONCOCT was able to do so as well. Additionally, *binny* performed also particularly well at recovering highly contiguous CAMI genomes. This can again be attributed to the ability to assess purity and completeness using the marker gene approach, here in particular for single-contig genomes. The marker gene approach in addition allows to bin also smaller contigs containing marker genes that would be discarded by most other binning methods due to their applied contig length thresholds. Although contigs below 1000 bp rarely made up more than 5% of the recovered MAGs size, *binny* assigned those usually with high precision (Supplementary Table 19).

binny also excels on co-assemblies, where the added information provided by contig depth of coverage data from multiple samples substantially increased performance. This is in line with previous studies observing additional discriminatory power of differential coverage depth compared to only sequence-based features [13, 15].

We also evaluated the effect of masking potentially disruptive sequence regions for the calculation of k-mer profiles. While the difference in performance with and without masking was not substantial, we believe that it reduces noise in the k-mer distributions of contigs from the same genome. One key reason for the small impact in the current setting might be the strong effect of the contig read depth on the embedding and clustering procedure, which could outweigh the impact of the masked k-mer profile. Masking reads mapping to the disruptive regions, also modifying the depth information might increase its effectiveness and could be implemented in future versions.

Finally, the results of the 105 metagenome benchmark shows that *binny*'s performance translates to real-world scenarios, massively outperforming other methods e.g. on the number of MIMAG High Quality MAGs recovered. Still, there are also many samples where all binning methods were unable to recover a sizeable proportion of the assemblies as MAGs of sufficient quality. This might hint at the still limited capabilities of binning methods or could be caused by low quality of these assemblies.

CONCLUSION

In conclusion, we demonstrate that *binny* outperforms currently available, state-of-the-art popular binning methods based on established evaluation metrics, recovering unique, high-quality MAGs from simple and complex samples alike, while being able to handle contiguous, as well as fragmented genomes. Moreover, we

could show that *binny* adds valuable new MAGs when used in combination with other binning methods and binning refinement approaches, enabling researchers to further improve the recovery of genomes from their metagenomes.

Additional file 1 — supplementary_figures.pdf

Contains supplementary figures 1-17.

Additional file 2 — supplementary_tables.xlsx

Contains supplementary tables 1-19 as Excel sheets.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

OH, PM, and AHB designed this study. OH and AHB created the application. OH performed all experiments. OH,PM and AHB wrote the manuscript; PQ and PW contributed to the review of the manuscript before submission. All authors read and approved the manuscript.

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Availability of data and material

The latest version of *binny* can be found at `https://github.com/a-h-b/binny`. Scripts used in this study and related data is available at `https://github.com/ohickl/binny_manuscript` and `https://doi.org/10.5281/zenodo.6577098`.

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