

MetaPhlAn2 for enhanced metagenomic taxonomic profiling

Duy Tin Truong¹, Eric Franzosa^{2,3}, Timothy L. Tickle^{2,3}, Matthias Scholz¹, George Weingart², Edoardo Pasolli¹, Adrian Tett¹, Curtis Huttenhower^{2,3}, and Nicola Segata¹

¹ Centre for Integrative Biology, University of Trento, Trento 38123, Italy

² Biostatistics Department, Harvard School of Public Health, Boston, Massachusetts 02115, USA

³ The Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, USA

Corresponding author: Nicola Segata, nicola.segata@unitn.it

MetaPhlAn¹ (Metagenomic Phylogenetic Analysis) is an efficient method to taxonomically characterize whole-metagenome shotgun (WMS) samples that has been successfully employed in large-scale microbial community studies^{2,3}. This work complements the original species-level profiling method with a system for eukaryotic and viral quantitation, strain-level identification, and strain tracking. These and other extensions make MetaPhlAn2 (<http://segatalab.cibio.unitn.it/tools/metaphlan2/>) an efficient method for mining WMS samples.

The method employs clade-specific markers to unequivocally assign reads to taxonomic clades, estimate these markers' coverages, and profile their presence and abundance¹. With a ~10× increase in sequenced genomes in the last two years, MetaPhlAn2 now includes ~1M markers (average 184 ± 45 for bacterial species) from >7,500 species (**Supplementary Tables 1–3**). Sub-species markers enable strain-level analyses, and quasi-markers improve accuracy and allow the detection of viruses and eukaryotic microbes (full list of additions in **Supplementary Notes 1–3**).

We validated MetaPhlAn2 using synthetic metagenomes (SMs, *Supplementary Note 4*). On the 24 SMs comprising 656 millions of reads and 1,295 species, MetaPhlAn2 proved more accurate than mOTU⁴ and Kraken⁵ (average correlation 0.95 ± 0.05 against 0.80 ± 0.21 and 0.75 ± 0.22, **Fig. 1a** and **Supplementary Figs. 1–8**) with fewer false positives–negatives (average 10–12 against 22–27 and 23–27), even when including genomes not in the reference database (see **Supplementary Note 4**). With the adoption of fast mappers (BowTie2) and support for parallelism, MetaPhlAn2 is >10× faster than MetaPhlAn1, and in line with the speed of other tested approaches (**Supplementary Fig. 9**).

We applied MetaPhlAn2 on four elbow skin samples we sequenced from three subjects (**Fig. 1b**, **Supplementary Note 5**). *Propionibacterium acnes* and *Staphylococcus epidermidis* dominate these sites, in agreement with expected genus-level results⁶ while providing species-level resolution. Together with these core species, we found *Malassezia globosa* in 93.65% of samples and confirmed it by coverage analysis (**Supplementary Fig. 10**). Although a known colonizer of the skin, its metagenomic characterization highlights the ability of MetaPhlAn2 in identifying non prokaryotic species. Phages (e.g., for *Propionibacterium*) as well as double-stranded DNA viruses of the Polyomavirus genus are also consistently detected. We then expanded the profiling to the whole set of 982 samples from other body sites from the HMP, including newly sequenced time points, as discussed in **Supplementary Note 6**.

Tracking microbes across samples has been performed extensively with culture-dependent approaches, and MetaPhlAn2 now offers this possibility in a culture-independent setting by fingerprinting the microbiome at the strain level. This is illustrated on the multiple time point ($n = 3$) HMP/HMP1-II dataset, in which species-specific strain fingerprints are subject-specific and conserved longitudinally (**Supplementary Note 7**). This both confirms strong subject-specific strain retention in the gut

microbiome and the ability of MetaPhlAn2 to perform strain fingerprinting/tracking, as such retention patterns are unlikely to occur in longitudinal samples by chance. Additionally, strain identification is available when a sample contains a previously sequenced genome (**Supplementary Note 7**). MetaPhlAn2's enhanced taxonomic profiling (including associated post-analysis, conversion, and visualization tools) and its characterization of the HMP/HMP1-II datasets should serve, respectively, as convenient tools and extended references for future analysis of the human microbiome.

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Competing financial interests

The authors declare no competing financial interests.

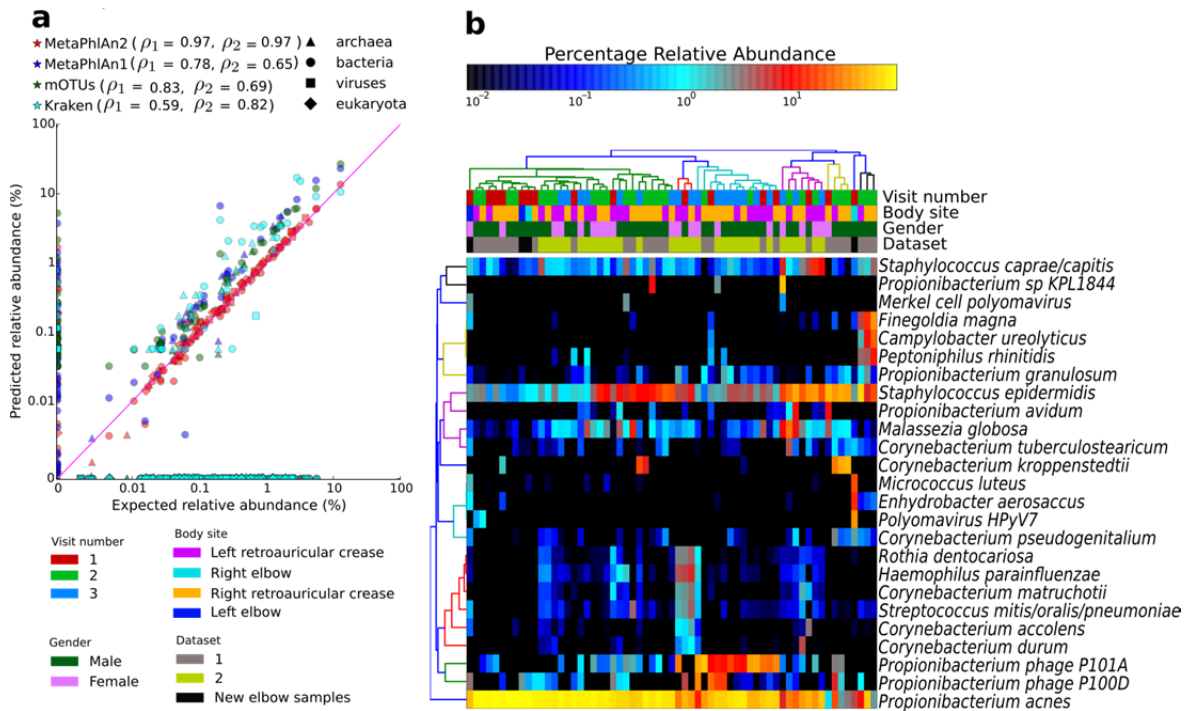
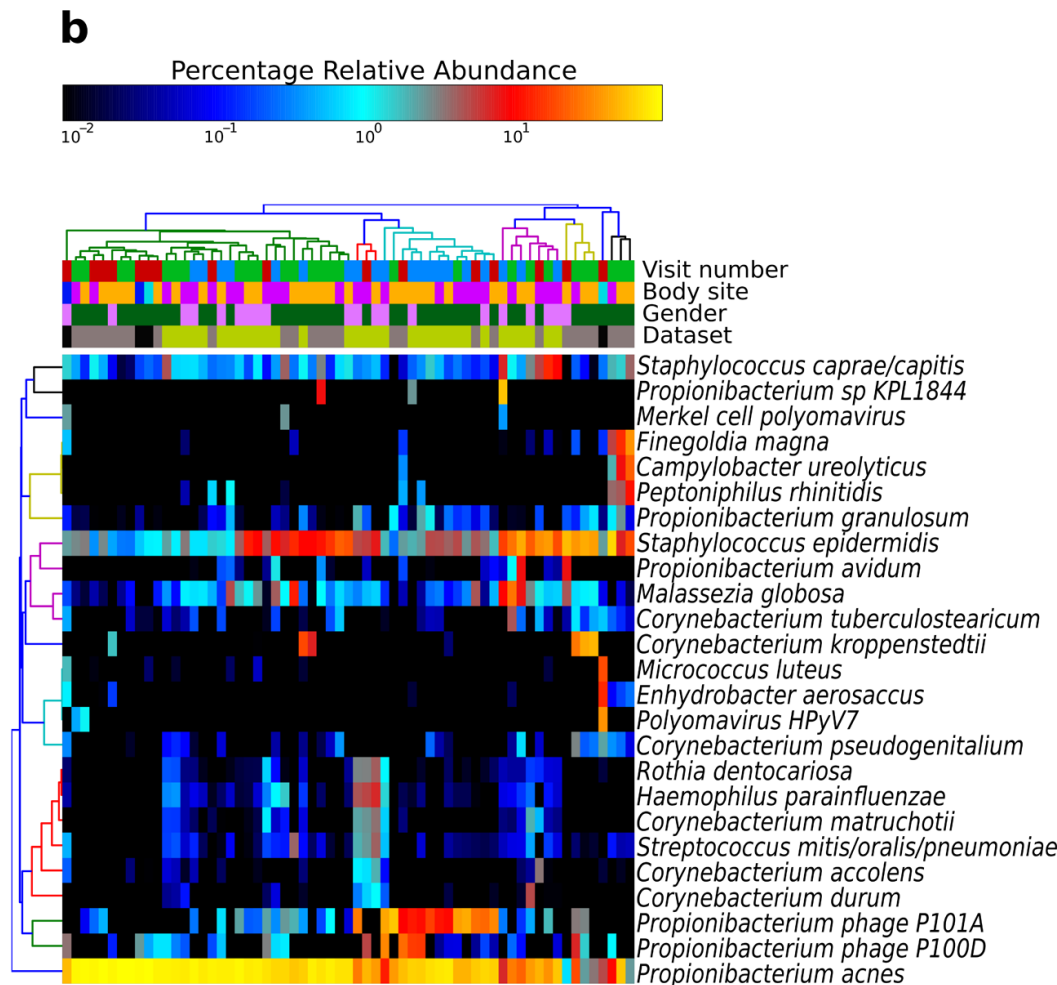
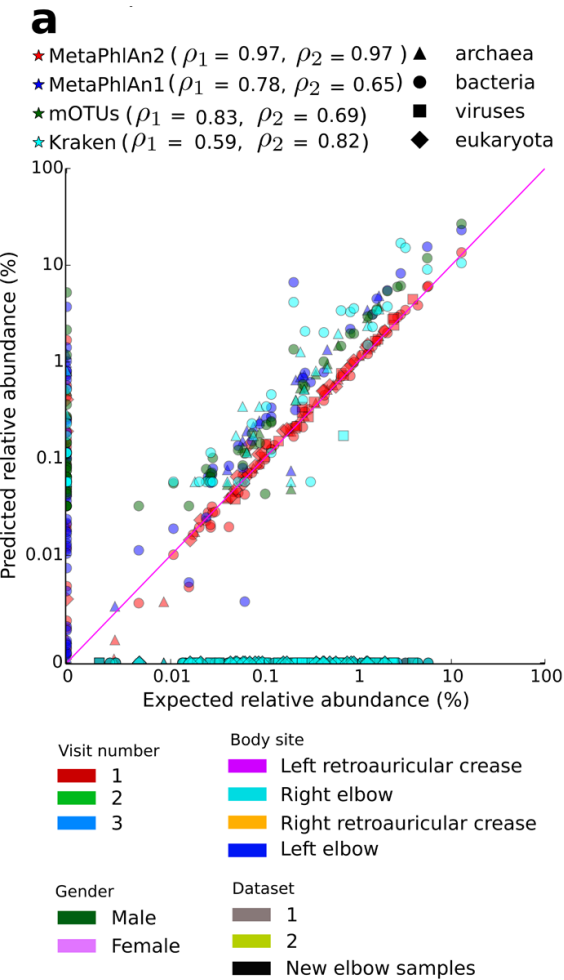


Figure 1. MetaPhlAn2 can accurately reconstruct the taxonomic composition of shotgun metagenomes. a) When tested on complex (40M 101 nt-long reads) metagenomes containing bacterial, archaeal, eukaryotic, and viral organisms (50, 25, 25 and 25 strains respectively) as well as 25 strains from unknown species (i.e., without reference genomes in the database), MetaPhlAn2 proved more accurate than MetaPhlAn1, mOTU and Kraken (for these three methods, correlations are reported for bacterial and archaeal organisms only as their support for viral and eukaryotic profiling is limited). b) MetaPhlAn2 characterization of all skin shotgun metagenomes available to date from the HMP and newly sequenced samples.

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Supplementary Note 1. Description of the main MetaPhlAn2 additions compared to MetaPhlAn1

- **Profiling of all domains of life.** Marker and quasi-marker genes are now identified not only for microbes (Bacteria and Archaea), but also for viruses and Eukaryotic microbes (Fungi, Protozoa) that are crucial components of microbial communities.
- **A 6-fold increase in the number of considered species.** Markers are now identified from >16,000 reference genomes and >7,000 unique species, dramatically expanding the comprehensiveness of the method. The new pipeline for identifying marker genes is also scalable to the quickly increasing number of reference genomes. See Supplementary Tables 1-3.
- **Introduction of the concept of quasi-markers, allowing more comprehensive and accurate profiling.** For species with less than 200 markers, MetaPhlAn2 adopts additional quasi-marker sequences (Supplementary Note 2) that are occasionally present in other genomes (because of vertical conservation or horizontal transfer). At profiling time, if no other markers of the potentially confounding species are detected, the corresponding quasi-local markers are used to improve the quality and accuracy of the profiling.
- **Addition of strain-specific barcoding for microbial strain tracking.** MetaPhlAn2 includes a completely new feature that exploits marker combinations to perform species-specific and genus-specific “barcoding” for strains in metagenomic samples (Supplementary Note 7). This feature can be used for culture-free pathogen tracking in epidemiology studies and strain tracking across microbiome samples. See Supplementary Figs. 12-20.
- **Strain-level identification for organisms with sequenced genomes.** For the case in which a microbiome includes strains that are very close to one of those already sequenced, MetaPhlAn2 is now able to identify such strains and readily reports their abundances. See Supplementary Note 7, Supplementary Table 13, and Supplementary Fig. 21.
- **Improvement of false positive and false negative rates.** Improvements in the underlying pipeline for identifying marker genes (including the increment of the adopted genomes and the use of quasi-markers) and the profiling procedure resulted in much improved quantitative performances (higher correlation with true abundances, lower false positive and false negative rates). See the validation on synthetic metagenomes in Supplementary Note 4.
- **Estimation of the percentage of reads mapped against known reference genomes.** MetaPhlAn2 is now able to estimate the number of reads that would map against genomes of each clade detected as present and for which an estimation of its relative abundance is provided by the default output. See Supplementary Note 3 for details.
- **Integration of MetaPhlAn with post-processing and visualization tools.** The MetaPhlAn2 package now includes a set of post-processing and visualization tools (“utils” subfolder of the MetaPhlAn2 repository). Multiple MetaPhlAn profiles can in fact be merged in an abundance table (“merge_metaphlan_tables.py”), exported as BIOM files, visualized as heatmap (“metaphlan_hclust_heatmap.py” or the integrated “hclust2” package), GraPhlAn plots (“export2graphlan.py” and the GraPhlAn package1), Krona2 plots (“metaphlan2krona.py”), and single microbe barplot across samples and conditions (“plot_bug.py”).

- **Cloud and Galaxy implementation for integrating MetaPhlAn in metagenomic pipelines.** MetaPhlAn2 is now conveniently available online in the Galaxy platform (e.g. at <http://huttenhower.sph.harvard.edu/galaxy>) and in the Galaxy Tool Shed and the obtained results can thus be readily post-processed with other Galaxy modules. MetaPhlAn2 is also natively included in cloud-based infrastructures such as Illumina BaseSpace.
- **Use of a fast DNA aligner (BowTie2).** MetaPhlAn2 dropped the direct support of the Blast suite, and is now focused on current high-speed read aligners, in particular BowTie2. This contributed to substantially improve the computational performances (Supplementary Fig. 9).
- **Support for parallelization and external mapping.** MetaPhlAn2 can exploit multiple threads with an almost linear speed-up (Supplementary Fig. 9). The metagenome mapping step can also be performed externally (e.g. by BowTie2) and the result then fed to MetaPhlAn2 as SAM files.
- **Added support for FastQ input files for more accurate mapping.** The per-base quality score included in FastQ formatted files are now used in the mapping procedure to improve the precision of the process.
- **Extended documentation with step-by-step tutorials.** Improved documentation and step-by-step tutorial (<http://segatalab.cibio.unitn.it/tools/metaphlan2/>) are now available to guide the user.
- **Python3, multiple input type (e.g. SAM), and piping support.** Python 3.x is now supported (in addition to Python 2.x) as well as non FastQ input files such as mapped SAM/BAMs. MetaPhlAn2 also support its inclusion in complex pipeline by accepting the input on the standard input and the used of named pipes.

Supplementary Note 2. Introduction of quasi-markers sequences in MetaPhlAn2

The selection of markers is performed by processing the available reference genomes (see Supplementary Tables 1 and 3) with a two-step procedure. First, for each clade, core genes are identified; then, in the second step, core genes with nontrivial homology with genomes from other clades are screened out. For the core gene identification step, the original strategy described for MetaPhlAn14 has been extended to robustly account for misannotated genomes, noisy gene calls, and inconsistencies in the underlying taxonomy as we described elsewhere^{5,6}. Additionally, we also now relax the uniqueness step by considering markers that show a minimal number of sequence hits in genomes outside the clade; such markers are called quasi-markers (see Supplementary Table 2), and their hits to external genomes are stored and used at profiling time. Specifically, a quasi-marker X for a clade A with an external hit to a genome of clade B is considered in estimating the relative abundance of A only if no other (strict) markers for clade B are present. Quasi-markers are ranked based on the number of external hits and are added to the marker set of a clade only if the number of (strict) markers is lower than 200. This allowed us to employ a larger number of markers for those clades with short genomes whose gene set is partially overlapping with other clades, and to be more robust to inconsistencies in the taxonomy or in the genome-associated information. Overall, the MetaPhlAn2 database includes 160,831 quasi-markers (18.3% of the total marker set) with avg 1.39 s.d. 17.2 external hits.

Supplementary Note 3. Estimating the percentage of reads mapped against known reference genomes

We introduced an estimation of the number of reads that would map against the genomes of clades with sequenced representatives. This estimation is enabled by the “-t rel_ab_w_read_stats” command line option. In brief, we estimate the RPKM (reads per kilo base per million mapped reads) assigned to each clade based on the (robustly computed) average RPKM of the markers using the core MetaPhlAn2 engine. Clade-specific RPKMs are then multiplied by the average genome length of the sequenced strains in the clade to obtain the average number of reads that would theoretically map against genomes of the clade. This is an interesting information that provides an estimate of the fraction of “microbial dark matter” in each sample without the need of an extensive and computationally unfeasible complete mapping of all reads against all available reference genomes. We illustrate the new this new MetaPhlAn2 features on the 763 HMP samples and 219 HMP11 samples (See Supplementary Note 6). The resulting predicted percentage of reads mapped against known reference genomes, is summarized in the boxplot of Supplementary Fig. 22. The median value on the entire set of samples is equal to 47%. Vaginal samples have the highest mappability (median above 90% for posterior fornix) due to the very high abundance of one of four vaginal *Lactobacillus* species with many sequenced genomes. Samples from the oral cavity and the skin have medians above 50% with the exception of the buccal mucosa. Gut samples have a rather small median value (28%), which is lower than the value of 42% found by extensive reads-to-genome mapping⁷. This is likely due to the fact that in [7] relatively permissive mapping parameters have been used, and to the fact that many reads from uncharacterized species are still mapping against conserved genomic regions of false positive species.

Supplementary Note 4. Validation of MetaPhlAn2 on synthetic metagenomes

Generation of synthetic metagenomes

We generated 22 synthetic metagenomes datasets of 10 or 40 millions of paired-end reads using SynMetaP8 comprising, in total, 482 bacterial, 80 archaeal, 331 viral, and 88 eukaryotic species. The synthetic metagenome generation was set to simulate Illumina HiSeq 101nt long reads, as the large majority of available metagenomic datasets have these characteristics. Half of these datasets were generated with an even distribution of species abundance, whereas for the other half we adopted a log-normal distribution of the abundances. The synthetic metagenomes also comprised a total of 48 genomes from species not present in the MetaPhlAn2 marker database in order to test the scenario in which the metagenomes include organisms without closely related sequenced genomes. We also included in the validation two

synthetic datasets available in literature⁹. The characteristics of all the datasets used are presented in Supplementary Table 5 and all the synthetic metagenomes are available at <http://goo.gl/5w9XTX>.

Comparative analysis of MetaPhlAn2, MetaPhlAn1, mOTU and Kraken

We compared MetaPhlAn2 with four other methods: MetaPhlAn14, mOTU¹⁰, Kraken¹¹ (mini-Kraken version), and Megan5¹². All methods were evaluated on all the generated synthetic metagenomes except for Megan that was applied on one sample only due to its high computational load. All methods were run with their default parameters. We assessed the performances of each method on each dataset using Pearson and Spearman correlation for log-normally distributed datasets, and root mean squared error for evenly distributed ones. In detail, Pearson correlation measures the linear correlation between two variables X and Y with value interval between +1 and -1, where 1 is total positive correlation, 0 is no correlation, and -1 is total negative correlation. Formally, Pearson correlation between two variables X and Y is defined as:

$$\rho_{X,Y} = \frac{\text{cov}(X,Y)}{\sigma_X \sigma_Y}$$

where $\text{cov}(X,Y)$ is the covariance between the two variables, and σ_X , σ_Y are the standard deviation of X and Y , respectively.

The Spearman correlation coefficient is the Pearson correlation between the ranked variables. For two variables X and Y with n raw scores X_i, Y_i , and the corresponding ranks x_i, y_i , the Spearman correlation is defined as:

$$\rho_{X,Y} = 1 - \frac{6 \sum (x_i - y_i)^2}{n(n^2 - 1)}$$

The root mean squared error measures the difference between the predicted and the true values of a variable. In detail, considered a variable Y and given n predictions \hat{y}_i and corresponding true values y_i , the root mean squared error is computed as:

$$\text{rmse}_{Y,\hat{Y}} = \sqrt{\frac{\sum (\hat{y}_i - y_i)^2}{n}}$$

Moreover, in this paper we counted as false positive (negative) the case in which the investigated method reports the presence (absence) of a species in the considered sample but is not really present (absent) based on the reference information. Supplementary Table 6 shows the average and standard deviation performance of four methods across 22 synthetic datasets. MetaPhlAn2 outperformed the other methods in terms of Pearson correlation, Spearman correlation and root mean squared error. Additionally, MetaPhlAn2 returned a smaller number of false positive and negative cases. A more detailed comparison is presented in Supplementary Tables 6-12 and Supplementary Figs. 1-8. The comparison with MEGAN5 (reported only for the sample Log_10M_1 in the Supplementary Table 9) showed how this tool is characterized by a low false negative rate at the price of a very high false positive rate and a prohibitive computational load.

Supplementary Note 5. Metagenomic sequencing of four new elbow metagenomic samples and their profiling with MetaPhlAn2

Sample collection, DNA extraction, and Illumina shotgun sequencing

Samples were collected by moistening cotton tip swabs (VWR, Milan, Italy) in SCF-1 sample buffer (50 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 8.0; 0.5% Tween-20) and swabbing the external elbow skin area for 30 seconds. To recover the sample the head of the swab was pushed against the side of sterile collection tube. Samples were pre-treated for 30 minutes at 37°C in a lysis solution (20 mM Tris-HCl, pH 8.0; 2 mM EDTA; 1 % Triton X-100) supplemented with Lysozyme (final concentration 20 mg/ml) (Sigma-Aldrich, Milan, Italy) before DNA was isolated with the Mo-Bio PowerSoil DNA

isolation kit (Mo Bio laboratories, Carlsband, CA,USA) as previously described¹³. Libraries were prepared using Illumina Nextera-XT DNA kit (Illumina inc, San Diego, CA, USA) as per manufacturer's instructions. Libraries were pooled and sequenced (101 bp paired end) on the Illumina HiSeq-2000 platform. The study was approved by the Ethical Committee of the University of Trento and informed written consent was obtained from all volunteers. The four samples have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession code 260277.

Sample preprocessing and MetaPhlan2 application

The four generated raw metagenomes were processed with FastqMcf¹⁴ by trimming positions with quality < 15, removing low-quality reads (mean quality < 25), and discarding reads shorter than 80 nt. We obtained 64 M reads (avg 16 M s.d. 6 M per sample) which were reduced to 44 M reads after human DNA and Bacteriophage phiX174 (Illumina spike-ins) removal (avg 11 M s.d. 7M per sample) by BowTie23 mapping against the reference genomes. MetaPhlan2 was then run on these samples with default parameters.

Supplementary Note 6. Application of MetaPhlan2 on the extended HMP/HMP-II dataset

We applied MetaPhlan2 with default parameters to 763 HMP samples and 219 HMP-II samples. The resulting profiled dataset are available on the HMP DACC (<http://hmpdacc.org/>) and at http://cibiocm.bitbucket.org/data/hmp_hmpii.tar.gz and visually summarized in the heatmap of Supplementary Fig. 11. Briefly, as described in¹⁵, stool samples were shotgun sequenced to a target depth of approximately 5-10 G nt per sample using 101nt Illumina GAIIx reads. Reads were quality trimmed, length filtered, and outlier samples quality controlled. See [13] for a full description of the methods.

Supplementary Note 7. Strain tracking and identification with MetaPhlan2

Strain-level markers are those genes that are uniquely present in the genome of that strain and absent from any other available genome (in the same or other species). These markers can be used to identify strains in the metagenomic samples that are very closely related to sequenced genomes. MetaPhlan2 performs this identification task by requiring that at least 70% of the markers available for a strain are present in the sample. When applied on real metagenomes, and specifically on the identification of *Bacteroides* strains in the human gut (from HMP samples), this resulted in the identification of strains that share at least 94% of the genome with the identified reference and with less than 0.5% SNP rates. As reported in Supplementary Table 13 and Supplementary Fig. 21, the other strains in the corresponding species have a much lower breadth of coverage (from 75% to 87% on average) and much higher SNP rates (above 1% in most cases). This confirmed the MetaPhlan2' ability to identify strains in the metagenomic sample that are very closely related with already sequenced strains. For the identified strains, a much deeper analysis of their strain-specific SNPs and functional repertoires can thus be performed using the identified genome as a reference for post-processing analyses.

Strain-level markers also provide a powerful means to perform strain barcoding and tracking. The set of strain-level markers belonging to a species can be seen as the subset of pan-genome genes for that species with the property of not being present in other species. Strains from the same species without sequenced representatives possess a combination of these species-specific pangenome markers that can thus be used to fingerprint them. By focusing on those markers that show variable patterns of presence/absence in different samples, it is then possible to estimate the genomic variability of sample-specific markers and, if patterns in different samples are extremely similar, to hypothesize microbial transmission (if samples belong to different subjects) or microbial retention (if samples belong to the same subject but collected at different time points).

To test this feature of MetaPhlan2, we generated twelve synthetic read sets (with the noise parameters described above) from six genomes (two sets for each genome at different coverage levels). The genomes were selected from two species (*Bacteroides fragilis* and *Bacteroides vulgatus*) and represented strains not already included in the generation of

the MetaPhlAn2 database and were thus ideal to test the ability of MetaPhlAn2 in typing and tracking previously unseen organisms. The twelve generated read sets were added to randomly-selected samples (Log_10M_1 to Log_10M_7). These combined samples were then profiled by MetaPhlAn2 and their barcoding strains are plotted as in Supplementary Figs. 19 and 20. MetaPhlAn2 successfully found (almost) identical barcodes for the same (unknown) strains shredded at different coverages and merged with different synthetic metagenomes. At the same time, different strains were clearly discriminated based on the presence/absence of their markers as highlighted by the dendrograms built using simple hierarchical clustering. The only case in which different strains were not clearly distinct in their barcodes occurred for two strains (Supplementary Fig. 20) that were indeed almost identical even looking at their original genome directly.

To further illustrate the strain tracking ability of MetaPhlAn2 on the extended HMP dataset, we plotted the presence/absence patterns of the markers from *Prevotella copri* (a known key member in the human gut, Supplementary Fig. 12) and from the three non-*Bacteroides* species and the three *Bacteroides* species most abundant in the human gut: *Alistipes putredinis* (Supplementary Fig. 13), *Eubacterium rectale* (Supplementary Fig. 14), *Parabacteroides merdae* (Supplementary Fig. 15), *Bacteroides ovatus* (Supplementary Fig. 16), *Bacteroides uniformis* (Supplementary Fig. 17), and *Bacteroides vulgatus* (Supplementary Fig. 18). By performing a hierarchical clustering (average linkage, hamming distance) and associating samples with subject IDs and collection time points, strong patterns of strain retention were evident (non-variable markers are removed from the visualization for improving readability).

Supplementary Table 1. Number of genomes in each domain of life used for MetaPhlAn2 marker definition

Kingdom	Number of genomes
Archaea	300
Bacteria	12926
Viruses	3565
Eukaryotes	112

Supplementary Table 2. Marker statistics in each domain of life considered in the MetaPhlAn2 database

Kingdom	Markers		Quasi markers		
	Total number	Avg. marker lengths (with s.d.)	Total number	Avg. marker lengths (with s.d.)	Avg. # of genomes violating the uniqueness property (with s.d.)
Archaea	46649	669.30 (526.78)	5613	728.81 (605.64)	9.34 (17.33)
Bacteria	767167	661.65 (541.89)	129614	689.03 (643.88)	6.62 (12.68)
Viruses	38809	688.86 (832.05)	23081	1028.60 (1454.94)	22.50 (107.54)
Euk.	22371	1156.54 (962.16)	2523	1141.27 (1336.81)	4.10 (9.21)

Supplementary Table 3. Number of distinct clades at different taxonomic levels considered in the MetaPhlAn2 database

Taxonomic levels	Number of different clades
Phyla	50
Classes	100
Orders	197
Families	481
Genera	1670
Species	7677
Species (excluding "spp.")	6500
Strains	16903

Supplementary Table 4. The number of reads in skin samples sequenced from three subjects

Skin samples	Total number of reads	Number of reads after quality control and removal of human DNA and Bacteriophage phiX174
Skin_1	11,781,066	2,878,998
Skin_2	9,481,814	5,643,960
Skin_3	25,653,734	21,789,989
Skin_4	16,751,638	13,486,694

Supplementary Table 5. The list and characteristics of the synthetic metagenomes used in this work

Synthetic metagenomes	Number of reads	Read length	Number of species	Abundance distribution
Even_10M_1	10 M	101	84	Evenly
Even_10M_2	10 M	101	87	Evenly
Even_10M_3	10 M	101	86	Evenly
Even_10M_4	10 M	101	89	Evenly
Even_10M_5	10 M	101	80	Evenly
Even_10M_6	10 M	101	88	Evenly
Even_10M_7	10 M	101	100	Evenly
Even_40M_1	40 M	101	150	Evenly
Even_40M_2	40 M	101	150	Evenly
Even_40M_3	40 M	101	150	Evenly
Even_40M_4	40 M	101	150	Evenly
Log_10M_1	10 M	101	85	log-normally
Log_10M_2	10 M	101	85	log-normally
Log_10M_3	10 M	101	85	log-normally
Log_10M_4	10 M	101	88	log-normally
Log_10M_5	10 M	101	92	log-normally
Log_10M_6	10 M	101	85	log-normally
Log_10M_7	10 M	101	100	log-normally
Log_40M_1	40 M	101	150	log-normally
Log_40M_2	40 M	101	150	log-normally
Log_40M_3	40 M	101	150	log-normally
Log_40M_4	40 M	101	150	log-normally

Supplementary Table 6. Average and standard deviation of the performances achieved by MetaPhlAn2, MetaPhlAn1, mOTUS and Kraken on the log-normally and evenly distributed datasets at the species level. The performance of MetaPhlAn2 is computed on four kingdoms (Archaeal, Bacterial, Viruses and Eukaryotic microbes) whereas the other methods are scored on Archaea and Bacteria only

	Method \ Dataset	Log datasets			Method \ Dataset	Even datasets		
		Average	S.d.			Average	S.d.	
Pearson correlation	MetaPhlAn2	0.95	0.05	Root mean squared error	MetaPhlAn2	0.34	0.08	
	MetaPhlAn1	0.80	0.21		MetaPhlAn1	1.20	0.25	
	mOTUs	0.80	0.21		mOTUs	1.10	0.24	
	Kraken	0.75	0.22		Kraken	1.61	0.44	
Spearman correlation	MetaPhlAn2	0.68	0.11		False positive	MetaPhlAn2	10	3
	MetaPhlAn1	0.18	0.18			MetaPhlAn1	25	9
	mOTUs	0.30	0.19			mOTUs	22	15
	Kraken	0.22	0.16			Kraken	23	10
False positive	MetaPhlAn2	13	7	False positive excluding "unclassified"	MetaPhlAn2	11	10	
	MetaPhlAn1	21	14		MetaPhlAn1	24	19	
	mOTUs	13	10		mOTUs	22	15	
	Kraken	20	12		Kraken	23	10	
False positive excluding "unclassified"	MetaPhlAn2	5	4	False negative	MetaPhlAn2	12	10	
	MetaPhlAn1	12	10		MetaPhlAn1	29	16	
	mOTUs	13	10		mOTUs	27	14	
	Kraken	20	12		Kraken	27	13	
False negative	MetaPhlAn2	33	10					
	MetaPhlAn1	35	15					
	mOTUs	33	13					
	Kraken	33	15					

Supplementary Table 7. Comparative results of the application of MetaPhlAn2, MetaPhlAn1, mOTUs, and Kraken on the log-normally distributed metagenomes in profiling the archaeal and bacterial organisms at the species level

	Method \ Dataset	Log 10M_1	Log 10M_2	Log 10M_3	Log 10M_4	Log 10M_5	Log 10M_6	Log 10M_7	Log 40M_1	Log 40M_2	Log 40M_3	Log 40M_4
Pearson correlation	MetaPhlAn2	0.99	1.00	0.99	0.98	0.90	0.97	1.00	0.97	0.96	0.79	1.00
	MetaPhlAn1	0.92	0.95	1.00	0.86	0.86	0.79	0.95	0.78	0.65	0.87	<0.50
	mOTUs	0.93	0.95	1.00	0.86	0.92	0.79	0.95	0.83	0.69	0.74	<0.50
	Kraken	0.84	0.93	1.00	0.81	0.88	0.76	0.67	0.59	0.82	0.82	<0.50
Spearman correlation	MetaPhlAn2	0.73	0.92	0.73	0.59	0.63	0.77	0.78	<0.50	0.55	0.86	0.88
	MetaPhlAn1	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50
	mOTUs	<0.50	0.66	0.52	<0.50	0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50
	Kraken	<0.50	0.51	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50
False positive	MetaPhlAn2	4	2	4	7	4	5	11	23	16	9	19
	MetaPhlAn1	12	11	6	7	11	9	23	47	30	31	41
	mOTUs	3	2	1	5	7	10	19	36	15	19	24
	Kraken	10	11	8	3	10	32	42	28	14	23	35
False positive excluding "unclassified"	MetaPhlAn2	0	0	0	0	1	1	4	13	5	2	7
	MetaPhlAn1	8	4	1	3	8	4	11	34	16	17	27
	mOTUs	3	2	1	5	7	10	19	36	15	19	24
	Kraken	10	11	8	3	10	32	42	28	14	23	35
False negative	MetaPhlAn2	17	12	14	21	13	16	22	27	33	5	6
	MetaPhlAn1	23	18	23	28	16	24	44	55	63	47	40
	mOTUs	26	16	26	28	19	24	42	52	58	42	31
	Kraken	21	17	22	26	19	21	44	54	59	48	37

Supplementary Table 8. Comparative results of the application of MetaPhlAn2 and Kraken on the log-normally distributed metagenomes in profiling the viral and eukaryotic organisms at the species level (the other methods are not able to detect viruses and eukaryote)

	Method \ Dataset	Log 10M_1	Log 10M_2	Log 10M_3	Log 10M_4	Log 10M_5	Log 10M_6	Log 10M_7
Pearson correlation	MetaPhlAn2	0.98	0.95	0.95	1.00	0.90	0.94	1.00
	Kraken	-0.02	0.89	0.00	0.98	0.75	0.72	0.89
Spearman correlation	MetaPhlAn2	0.37	0.71	0.57	0.74	0.38	0.73	0.76
	Kraken	0.17	0.38	-0.16	0.24	0.35	0.33	0.51
False positive	MetaPhlAn2	7	3	4	1	5	2	2
	Kraken	0	0	2	1	0	0	0
False positive excluding "unclassified"	MetaPhlAn2	5	1	2	0	2	1	1
	Kraken	0	0	2	1	0	0	0
False negative	MetaPhlAn2	22	18	25	22	24	21	5
	Kraken	39	39	45	42	45	41	19

Supplementary Table 9. Comparative results of the application of MetaPhlAn2, MetaPhlAn1, mOTUs, Kraken, and MEGAN5 on the log-normally distributed metagenomes in profiling the archaeal, bacterial, viral and eukaryotic organisms at the species level

	Method \ Dataset	Log 10M_1	Log 10M_2	Log 10M_3	Log 10M_4	Log 10M_5	Log 10M_6	Log 10M_7	Log 40M_1	Log 40M_2	Log 40M_3	Log 40M_4
Pearson correlation	MetaPhlAn2	0.98	0.95	0.99	1.00	0.90	0.94	1.00	0.97	0.97	0.81	0.99
	MetaPhlAn1	<0.50	<0.50	1.00	<0.50	0.86	0.73	0.91	0.72	<0.50	0.86	<0.50
	mOTUs	<0.50	<0.50	1.00	<0.50	0.91	0.74	0.91	0.77	<0.50	0.73	<0.50
	Kraken	<0.50	<0.50	1.00	0.61	0.88	0.70	0.64	0.55	<0.50	0.80	<0.50
	MEGAN5	0.84	-	-	-	-	-	-	-	-	-	-
Spearman correlation	MetaPhlAn2	0.57	0.80	0.64	0.66	0.53	0.75	0.78	0.56	0.61	0.78	0.85
	MetaPhlAn1	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50
	mOTUs	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50
	Kraken	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50
	MEGAN5	<0.50	-	-	-	-	-	-	-	-	-	-
False positive	MetaPhlAn2	11	5	8	8	9	7	13	26	20	14	22
	MetaPhlAn1	12	11	6	7	11	9	23	47	30	31	41
	mOTUs	3	2	1	5	7	10	19	36	15	19	24
	Kraken	10	11	10	4	10	32	42	29	14	23	36
	MEGAN5	>100	-	-	-	-	-	-	-	-	-	-
False positive excluding "unclassified"	MetaPhlAn2	5	1	2	0	3	2	5	13	5	5	9
	MetaPhlAn1	8	4	1	3	8	4	11	34	16	17	27
	mOTUs	3	2	1	5	7	10	19	36	15	19	24
	Kraken	10	11	10	4	10	32	42	29	14	23	36
	MEGAN5	>100	-	-	-	-	-	-	-	-	-	-
False negative	MetaPhlAn2	39	30	39	43	37	37	27	35	47	22	10
	MetaPhlAn1	63	61	69	73	64	67	65	105	113	97	90
	mOTUs	66	59	72	73	67	67	63	102	108	92	81
	Kraken	60	56	67	68	64	62	63	103	106	96	83
	MEGAN5	8	-	-	-	-	-	-	-	-	-	-

Supplementary Table 10. Comparative results of the application of MetaPhlAn2, MetaPhlAn1, mOTUs, and Kraken on the evenly distributed metagenomes in profiling the archaeal and bacterial organisms at the species level

	Method \ Dataset	Even 10M_1	Even 10M_2	Even 10M_3	Even 10M_4	Even 10M_5	Even 10M_6	Even 10M_7	Even 40M_1	Even 40M_2	Even 40M_3	Even 40M_4
Root mean square error	MetaPhlAn2	0.59	0.39	0.51	0.63	0.40	0.35	0.30	0.54	0.56	0.19	0.27
	MetaPhlAn1	1.39	1.43	1.47	1.40	1.37	1.44	1.09	0.96	0.98	0.85	0.79
	mOTUs	1.30	1.43	1.35	1.21	1.28	1.26	0.93	0.90	0.92	0.78	0.72
	Kraken	2.07	2.02	2.02	1.93	1.85	2.12	1.42	1.20	1.11	0.99	1.03
False positive	MetaPhlAn2	6	7	6	5	8	6	11	17	11	3	7
	MetaPhlAn1	13	25	16	18	18	27	23	44	26	27	35
	mOTUs	6	20	6	8	15	13	21	56	43	28	27
	Kraken	12	30	9	12	14	23	34	39	35	20	24
False positive excluding "unclassified"	MetaPhlAn2	3	2	2	1	3	2	6	29	22	11	17
	MetaPhlAn1	6	15	5	9	9	16	13	61	43	41	50
	mOTUs	6	20	6	8	15	13	21	57	44	28	27
	Kraken	12	30	9	12	14	23	34	39	35	20	24
False negative	MetaPhlAn2	2	1	1	1	1	1	2	26	26	2	3
	MetaPhlAn1	14	19	13	15	14	22	25	54	56	44	38
	mOTUs	13	19	16	11	15	20	26	51	52	39	30
	Kraken	11	20	14	16	13	22	30	48	50	35	34

Supplementary Table 11. Comparative results of the application of MetaPhlAn2 and Kraken on the evenly distributed metagenomes in profiling the viral and eukaryotic organisms at the species level

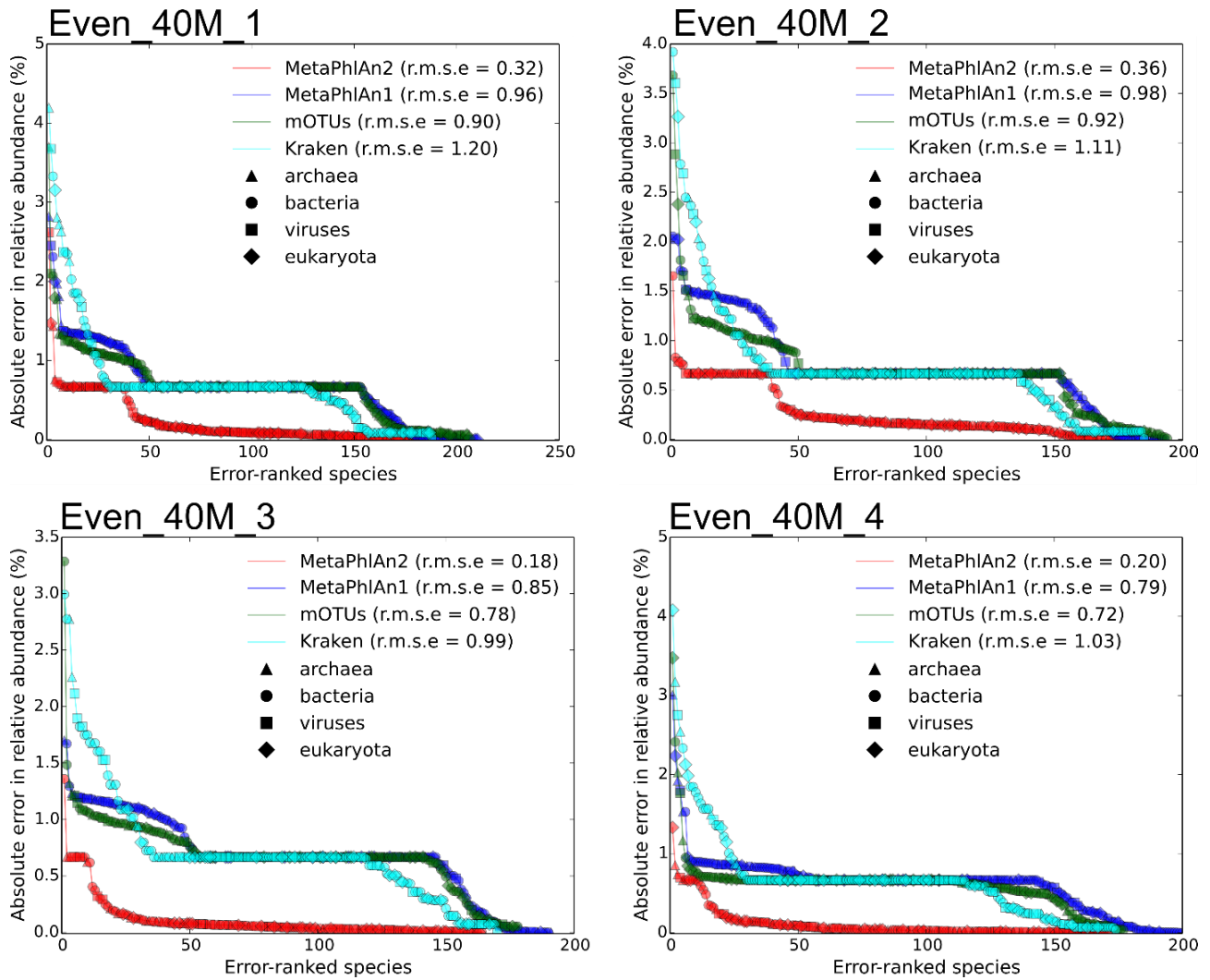
	Method \ Dataset	Even 10M_1	Even 10M_2	Even 10M_3	Even 10M_4	Even 10M_5	Even 10M_6	Even 10M_7
Root mean square error	MetaPhlAn2	1.20	1.22	1.13	1.04	1.32	0.98	1.39
	Kraken	11.13	15.43	7.67	14.58	11.66	15.81	12.67
False positive	MetaPhlAn2	5	2	4	4	4	2	3
	Kraken	0	0	0	0	0	0	0
False positive excluding "unclassified"	MetaPhlAn2	1	1	1	1	2	0	0
	Kraken	0	0	0	0	0	0	0
False negative	MetaPhlAn2	8	6	7	8	7	4	2
	Kraken	41	40	40	45	37	38	27

Supplementary Table 12. Comparative results of the application of MetaPhlAn2, MetaPhlAn1, mOTUs, and Kraken on the evenly distributed metagenomes in profiling the archaeal, bacterial, viral and eukaryotic organisms at the species level

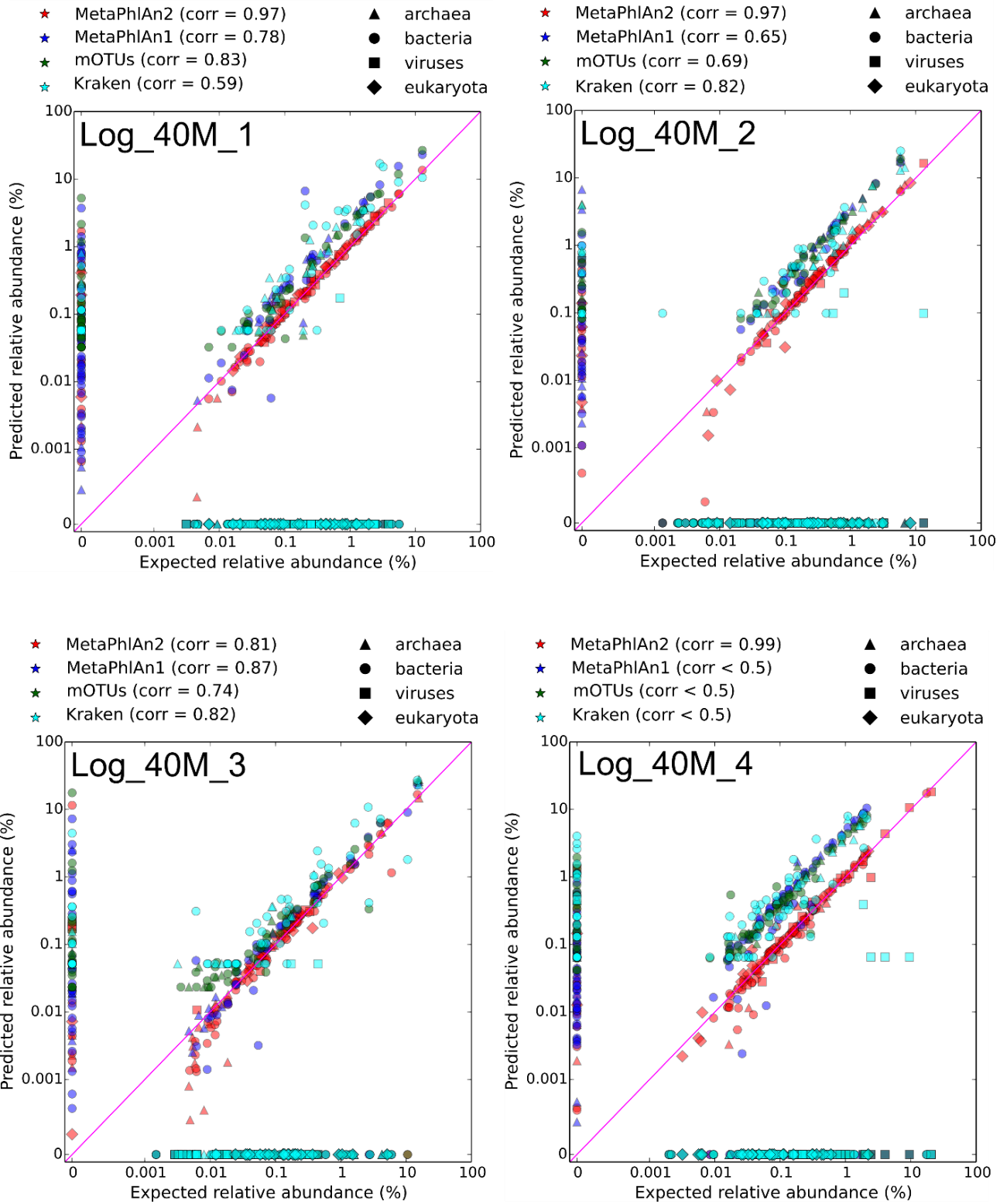
	Method \ Dataset	Even 10M_1	Even 10M_2	Even 10M_3	Even 10M_4	Even 10M_5	Even 10M_6	Even 10M_7	Even 40M_1	Even 40M_2	Even 40M_3	Even 40M_4
Root mean square error	MetaPhlAn2	0.44	0.38	0.41	0.42	0.39	0.32	0.32	0.32	0.36	0.18	0.20
	MetaPhlAn1	1.75	1.52	1.59	1.48	1.73	1.52	1.07	0.86	0.89	0.81	0.75
	mOTUs	1.77	1.42	1.53	1.45	1.70	1.38	0.92	0.82	0.84	0.77	0.73
	Kraken	1.79	1.79	1.88	1.84	1.84	1.96	1.32	1.06	0.99	0.92	0.95
False positive	MetaPhlAn2	11	9	10	9	12	8	14	17	12	4	9
	MetaPhlAn1	13	25	16	18	18	27	23	44	26	27	35
	mOTUs	6	20	6	8	15	13	21	56	43	28	27
	Kraken	12	30	9	12	14	23	34	39	35	20	24
False positive excluding "unclassified"	MetaPhlAn2	4	3	3	2	5	2	6	32	26	14	21
	MetaPhlAn1	6	15	5	9	9	16	13	61	43	41	50
	mOTUs	6	20	6	8	15	13	21	57	44	28	27
	Kraken	12	30	9	12	14	23	34	39	35	20	24
False negative	MetaPhlAn2	10	7	8	9	8	5	4	30	34	8	6
	MetaPhlAn1	57	60	57	61	53	61	54	104	106	94	88
	mOTUs	56	60	60	57	54	59	55	101	102	89	80
	Kraken	52	60	54	61	50	60	57	96	98	83	82

Supplementary Table 13. An example of strain identification for *Bacteroides* strains on the gut HMP samples. The samples in which MetaPhlAn2 consistently detects a given *Bacteroides* strain are reported and complemented for validation purposes with their breadth of coverage (i.e., percentage of the strain genome covered by reads) and the best and average (with s.d.) breadth of coverage for all the other available genomes in the same species. The number of single nucleotide polymorphism (SNPs, by comparison of mapping consensus with the reference genome) are also reported for the detected strain and all the other strains in the species

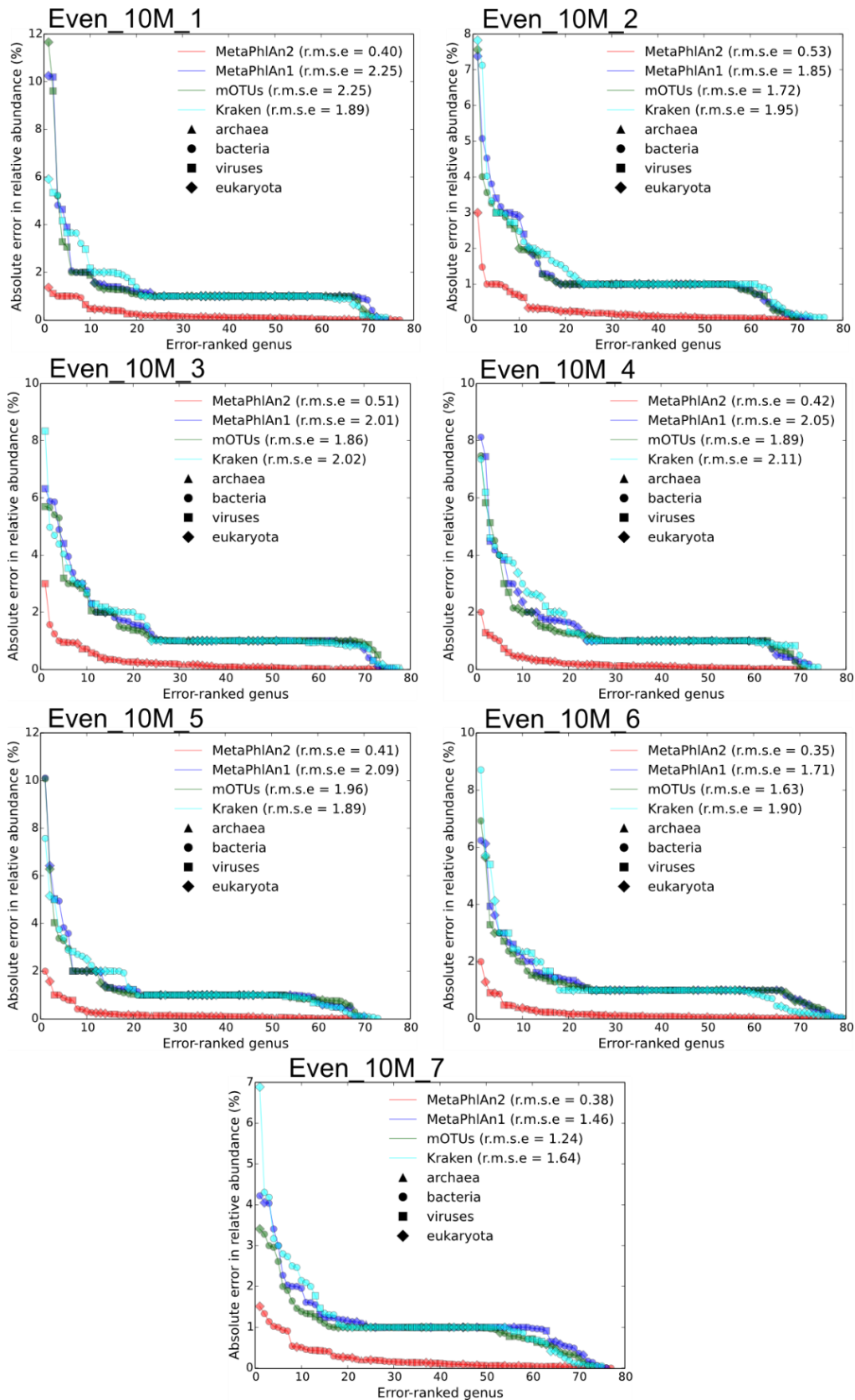
Sample ID	Identified Strain	Cov. breadth	Best breadth (other strains)	Avg. breadth (other strains)	# of SNPs	Min # SNPs (other strains)	Avg. # of SNPs (other strains)
SRS011586	<i>B. fingoldii</i> DSM 17565	0.94	0.75	0.75±0.00	22131	106486	106829±343
SRS011586	<i>B. fragilis</i> HMW 616	0.94	0.85	0.57±0.11	33002	64117	186206±52058
SRS011586	<i>B. uniformis</i> ATCC 8492	0.95	0.81	0.79±0.02	8700	52449	56640±5548
SRS012273	<i>B. ovatus</i> CL03T12C18	0.92	0.78	0.76±0.02	80767	148729	160700±9318
SRS012273	<i>B. uniformis</i> ATCC 8492	0.94	0.79	0.78±0.01	17182	62025	65538±4482
SRS014683	<i>B. ovatus</i> CL03T12C18	0.94	0.85	0.83±0.02	14223	101081	114476±12741
SRS015133	<i>B. fragilis</i> HMW 615	0.95	0.87	0.76±0.18	15343	41570	70400±49714
SRS016203	<i>B. uniformis</i> ATCC 8492	0.98	0.81	0.79±0.03	10647	53407	57115±4348
SRS016267	<i>B. ovatus</i> SD CMC 3f	0.97	0.79	0.76±0.03	11576	102563	111011±6418
SRS019030	<i>B. ovatus</i> CL03T12C18	0.94	0.87	0.85±0.02	17440	104773	117105±11462
SRS019787	<i>B. ovatus</i> SD CMC 3f	0.97	0.79	0.76±0.03	8224	110630	117565±6295
SRS049900	<i>B. uniformis</i> ATCC 8492	0.96	0.79	0.76±0.04	28353	67084	70203±2270
SRS064645	<i>B. uniformis</i> ATCC 8492	0.96	0.78	0.77±0.02	15463	57678	60234±2765



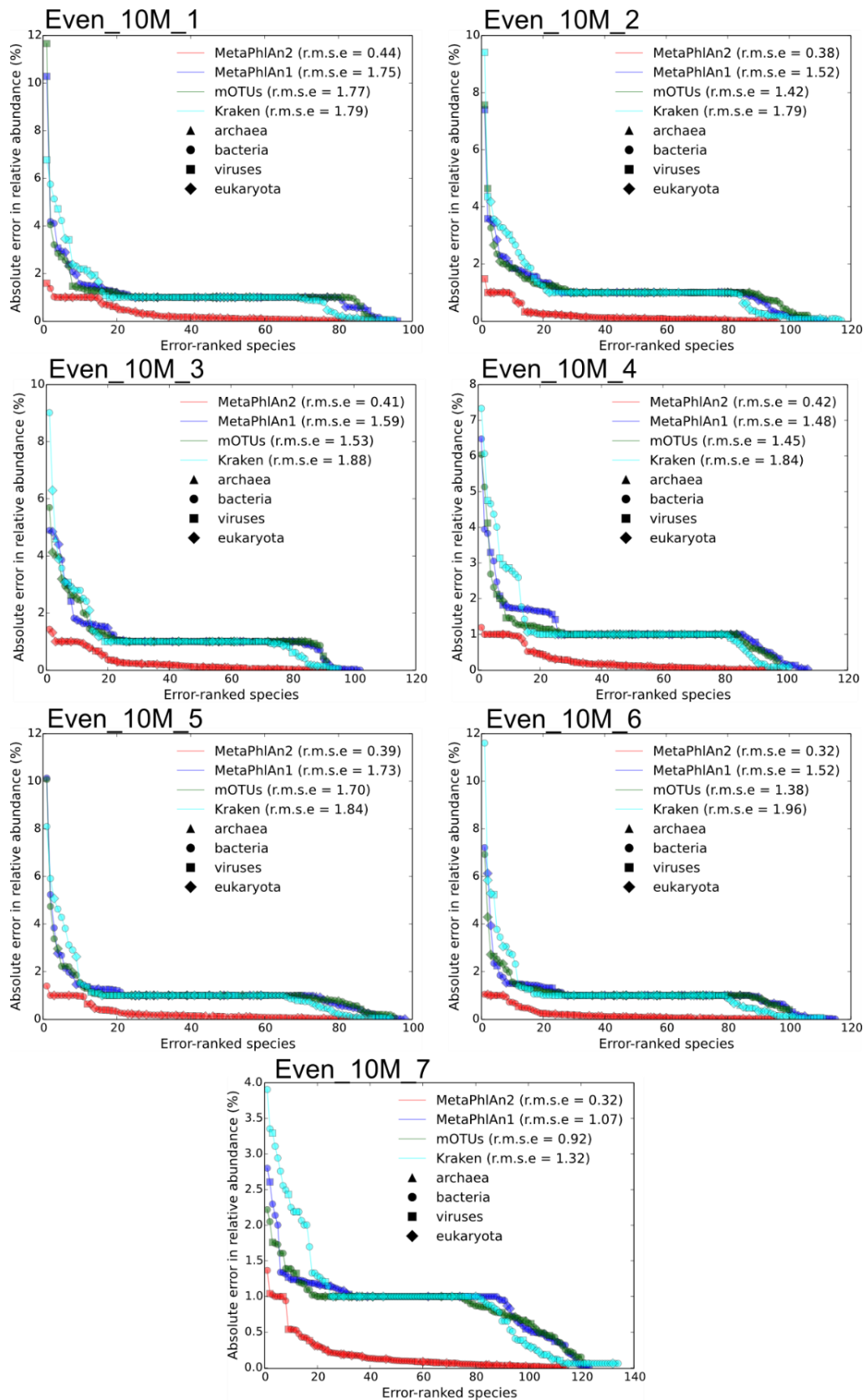
Supplementary Fig. 1. Performance comparison of the four tested methods on evenly distributed 40M-read datasets at the species level based on the ranked root mean squared error (r.m.s.e). The performance of MetaPhlAn2 is computed on four kingdoms (Archaeal, Bacterial, Viruses and Eukaryotic microbes) whereas the other methods are scored on Archaea and Bacteria only



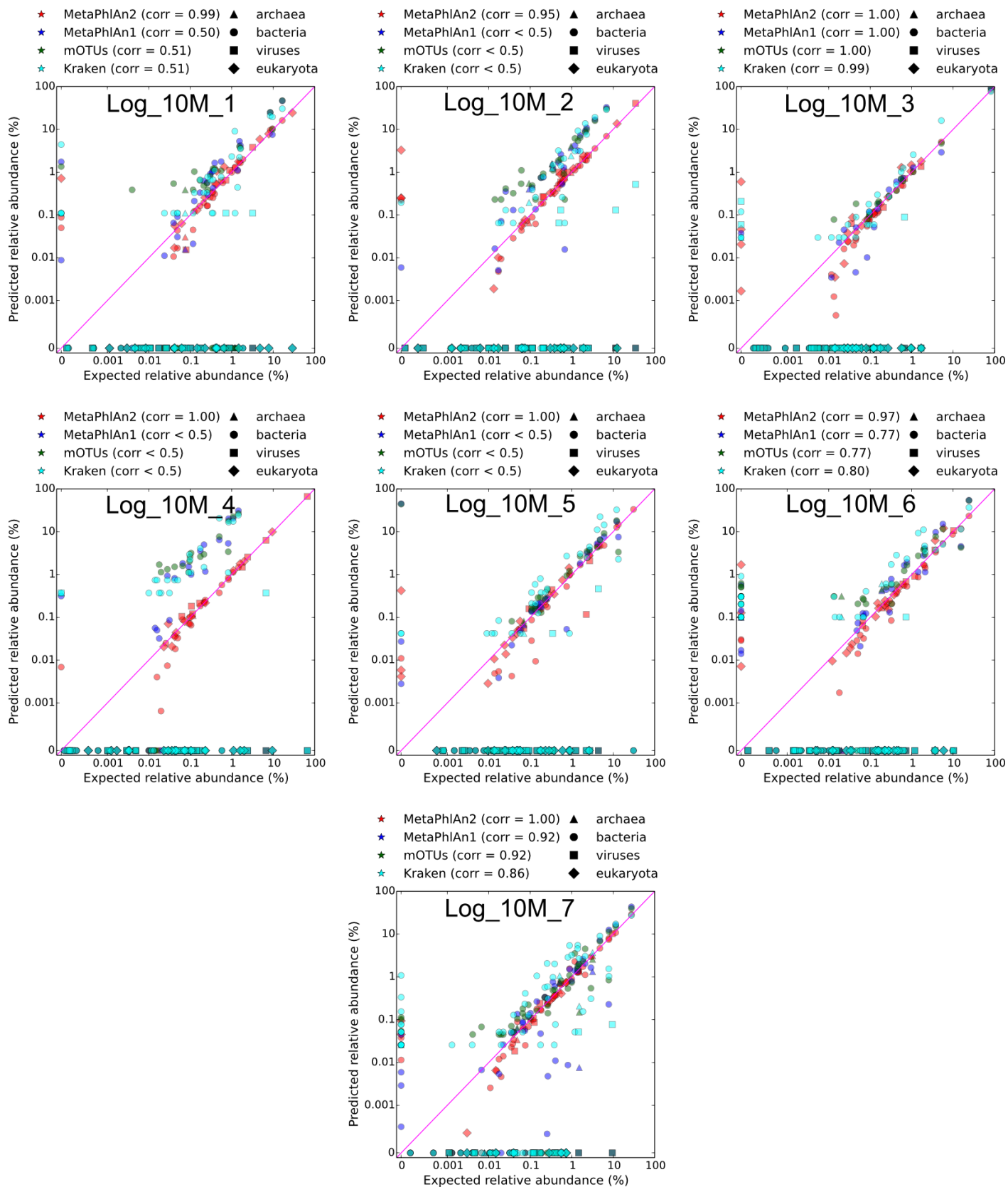
Supplementary Fig. 2. Performance comparison of the four tested methods on log-normally distributed 40M-read datasets at the species level based on the Pearson correlation (corr). The performance of MetaPhlAn2 is computed on four kingdoms (Archaeal, Bacterial, Viruses and Eukaryotic microbes) whereas the other methods are scored on Archaea and Bacteria only



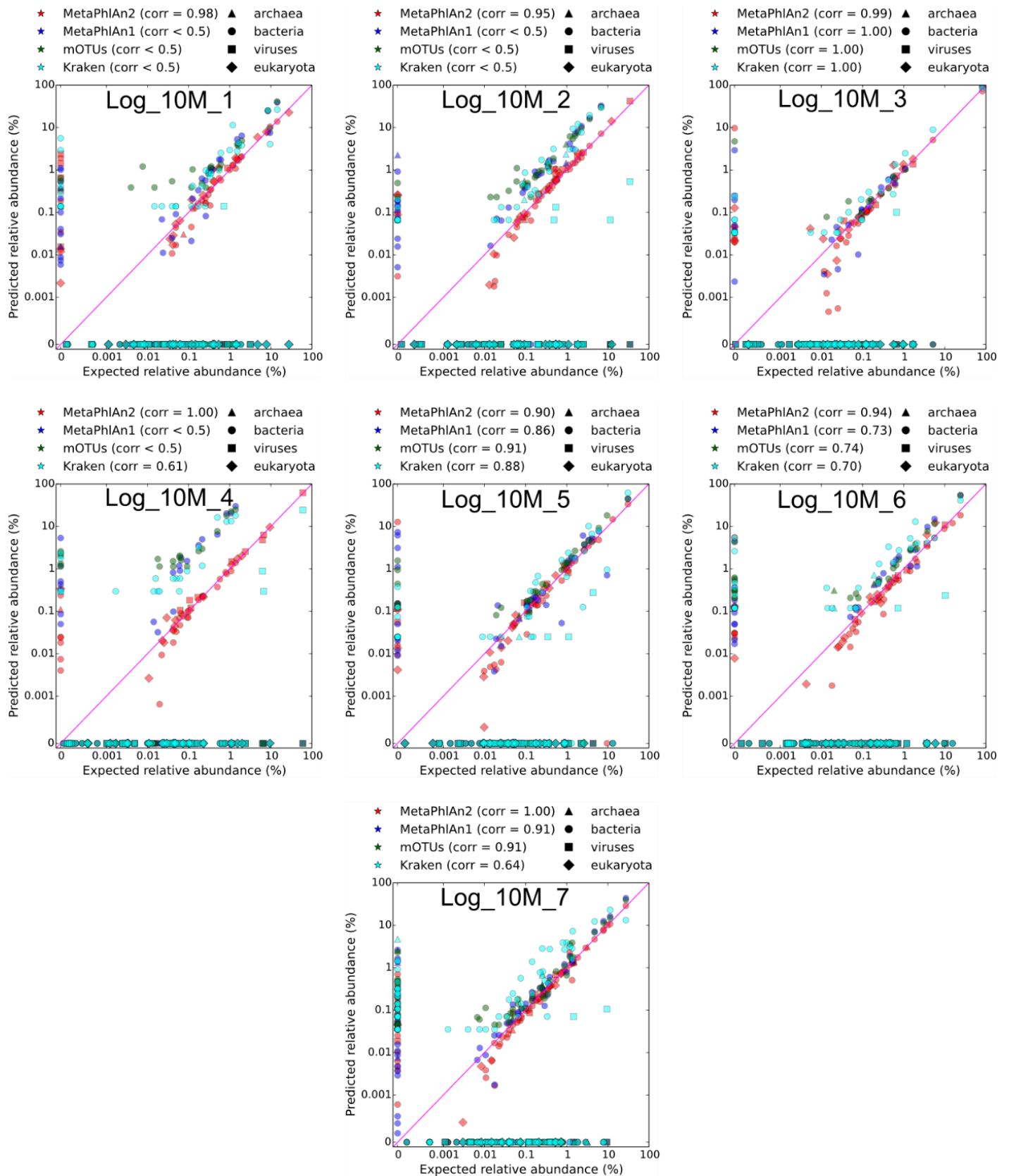
Supplementary Fig. 3. Performance comparison of the four tested methods on evenly distributed 10M-read datasets at the genus level based on the ranked root mean squared error (r.m.s.e). The performance of all methods are computed on four kingdoms (Archaeal, Bacterial, Viruses and Eukaryotic microbes)



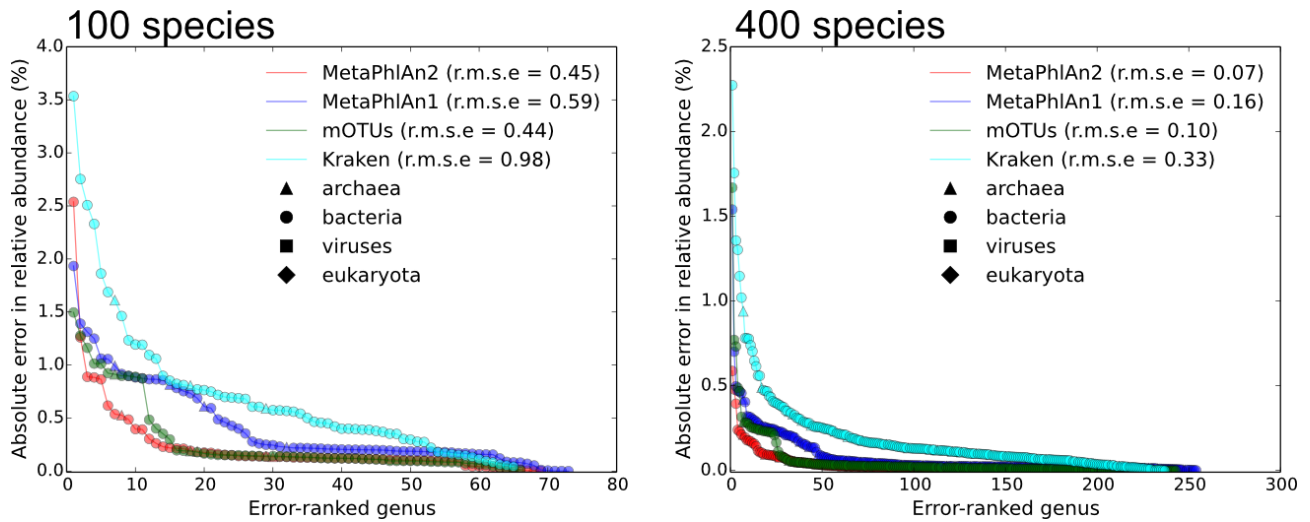
Supplementary Fig. 4. Performance comparison of the four tested methods on evenly distributed 10M-read datasets at the species level based on the ranked root mean squared error (r.m.s.e). The performance of all methods are computed on four kingdoms (Archaeal, Bacterial, Viruses and Eukaryotic microbes)



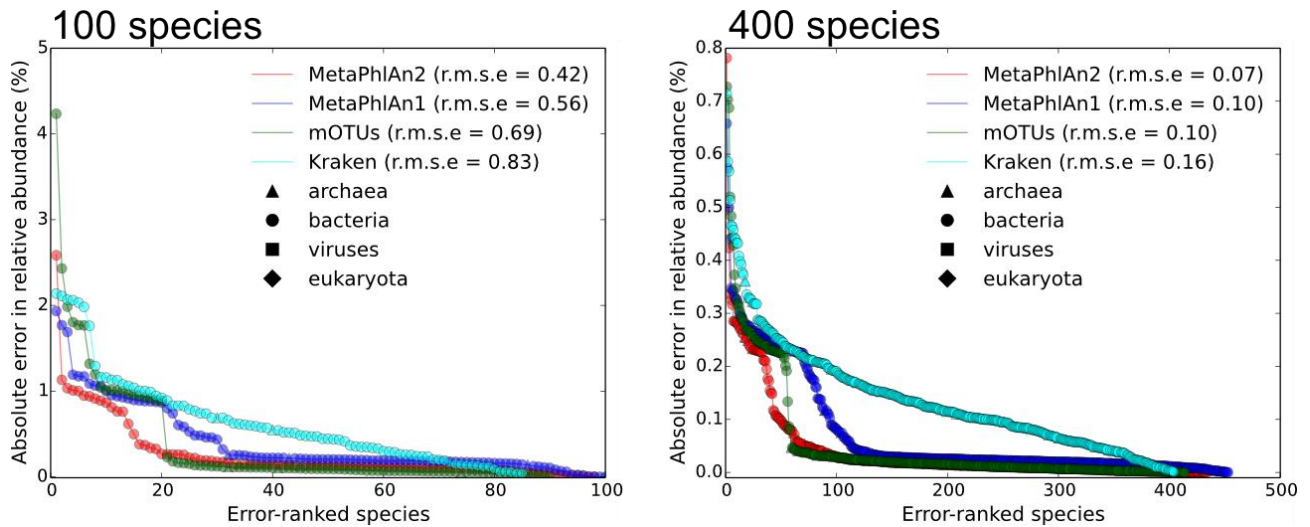
Supplementary Fig. 5. Performance comparison of the four tested methods on log-normally distributed 10M-read datasets at the genus level based on the Pearson correlation (corr). The performance of all methods are computed on four kingdoms (Archaeal, Bacterial, Viruses and Eukaryotic microbes)



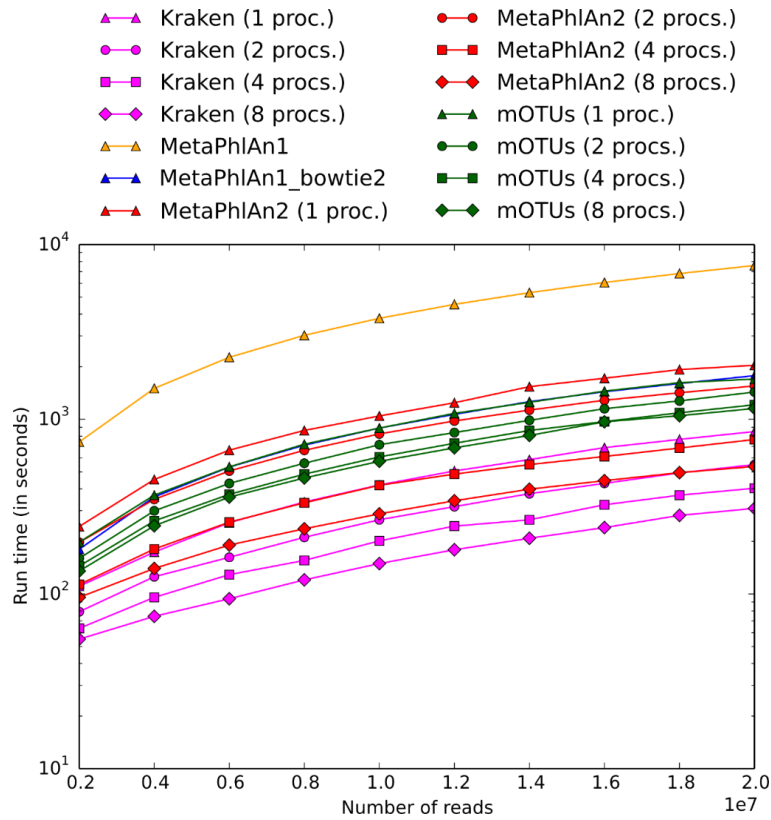
Supplementary Fig. 6. Performance comparison of the four tested methods on log-normally distributed 10M-read datasets at the species level based on the Pearson correlation (corr). The performance of all methods are computed on four kingdoms (Archaeal, Bacterial, Viruses and Eukaryotic microbes)



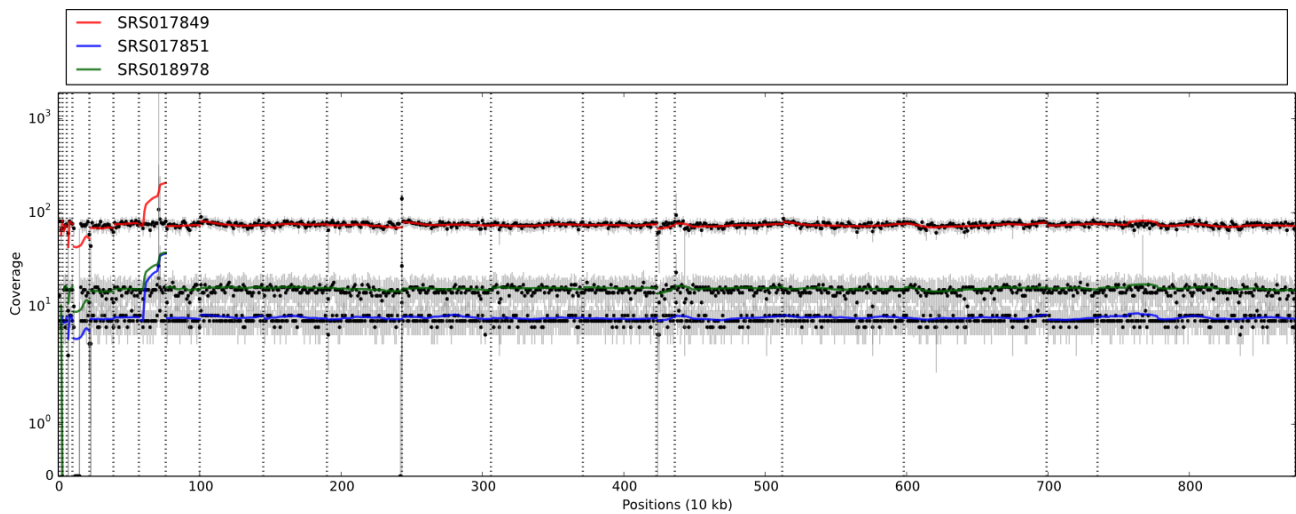
Supplementary Fig. 7. Performance comparison of the four tested methods on the Mende *et al.*'s datasets9 at the genus level based on the ranked root mean squared error (r.m.s.e). The performance of all methods are computed on four kingdoms (Archaeal, Bacterial, Viruses and Eukaryotic microbes)



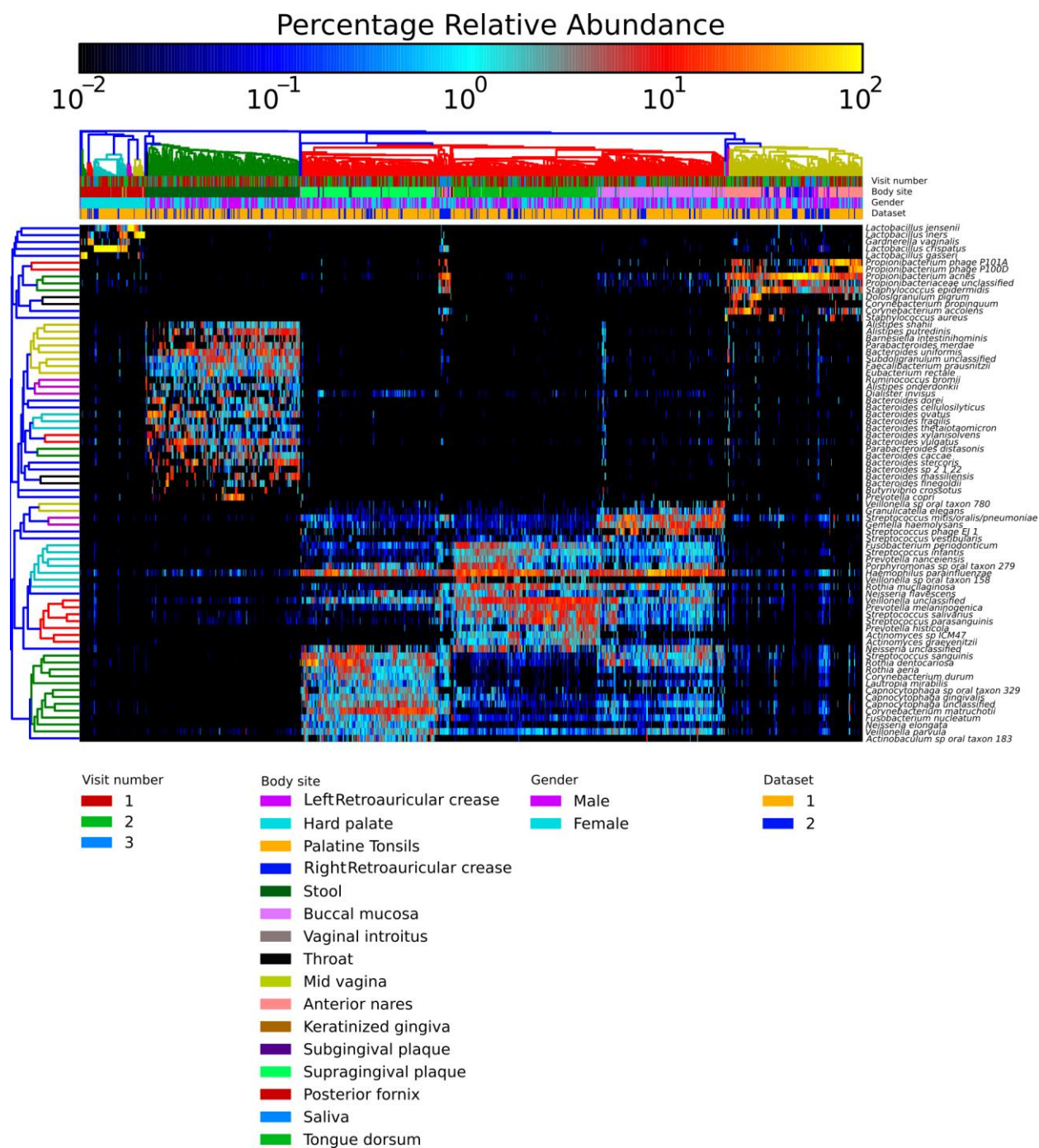
Supplementary Fig. 8. Performance comparison of the four tested methods on the Mende *et al.*'s datasets9 at the species level based on the ranked root mean squared error (r.m.s.e). The performance of all methods are computed on four kingdoms (Archaea, Bacteria, Viruses and Eukaryotes)



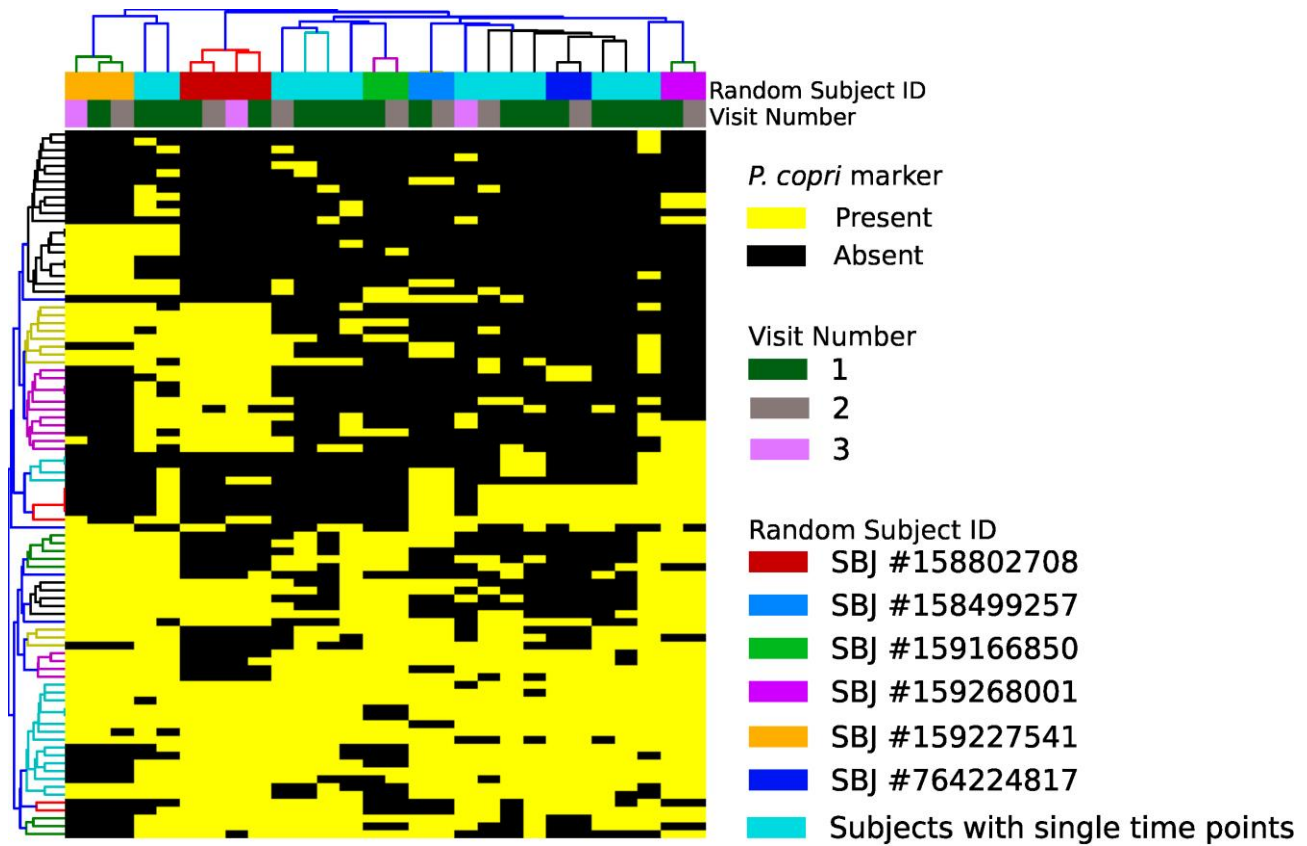
Supplementary Fig. 9. Run-time comparison between the validated methods. The original implementation of MetaPhlAn14 was based on Blastn¹⁶, but we evaluate here also its extension based on BowTie23. MetaPhlAn2, mOTUS, and Kraken are evaluated at increasing number of processors (from 1 to 8)



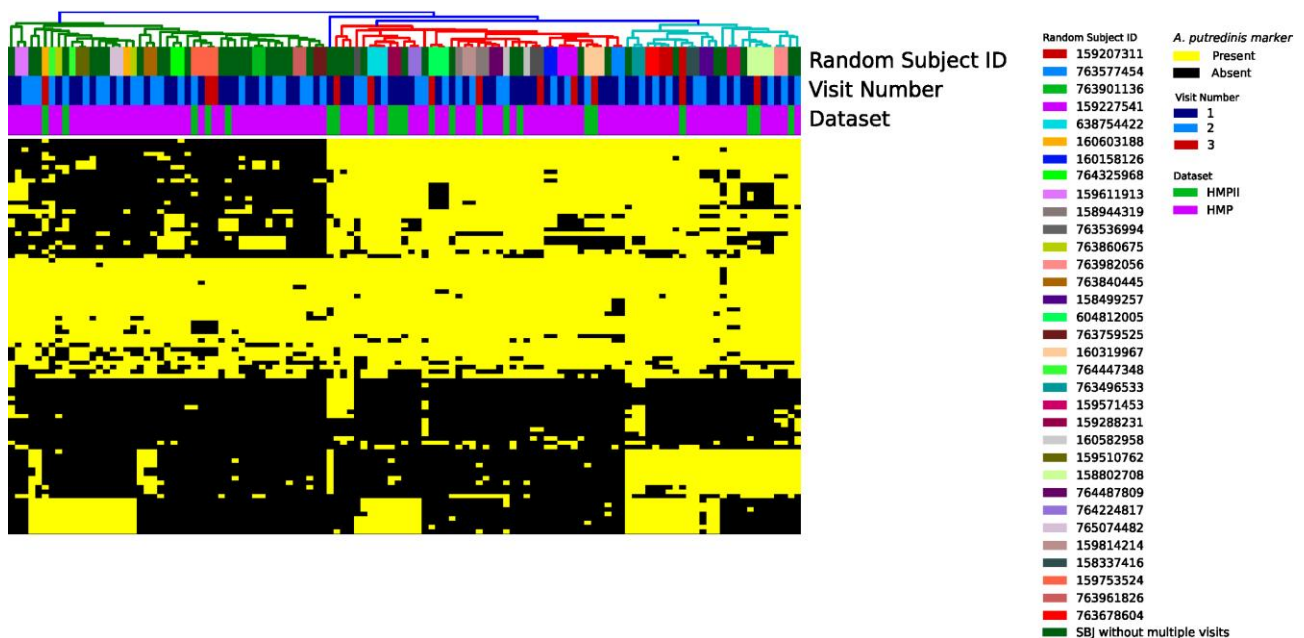
Supplementary Fig. 10. Genome coverage plots of three HMP samples against the reference genome of *Malassezia globosa* (GCA_000181695) confirm the presence of this eukaryotic microbe on the human skin. Each point reports the average coverage on 10 kb windows, whereas the gray bars display the interquartile ranges



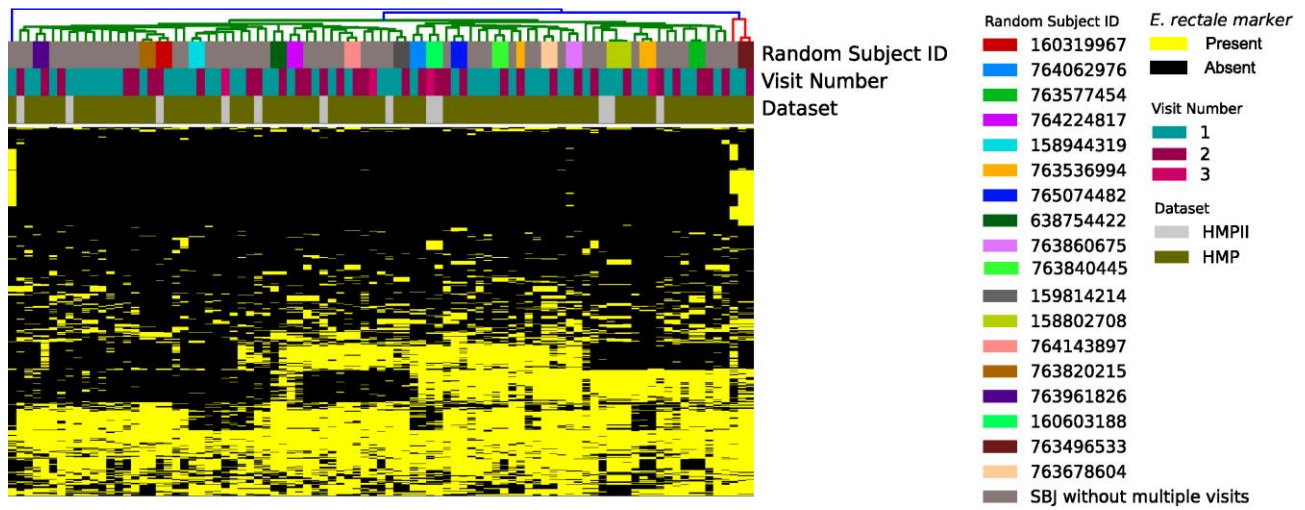
Supplementary Fig. 11. MetaPhlan2 profiling of HMP and HMPII samples. Only the 75 most abundant species (according to the 99th percentile ranking) are reported. Microbial species and samples are hierarchically clustered (average linkage) using correlation and Bray-Curtis distance (in root square abundance spaces) as similarity functions respectively



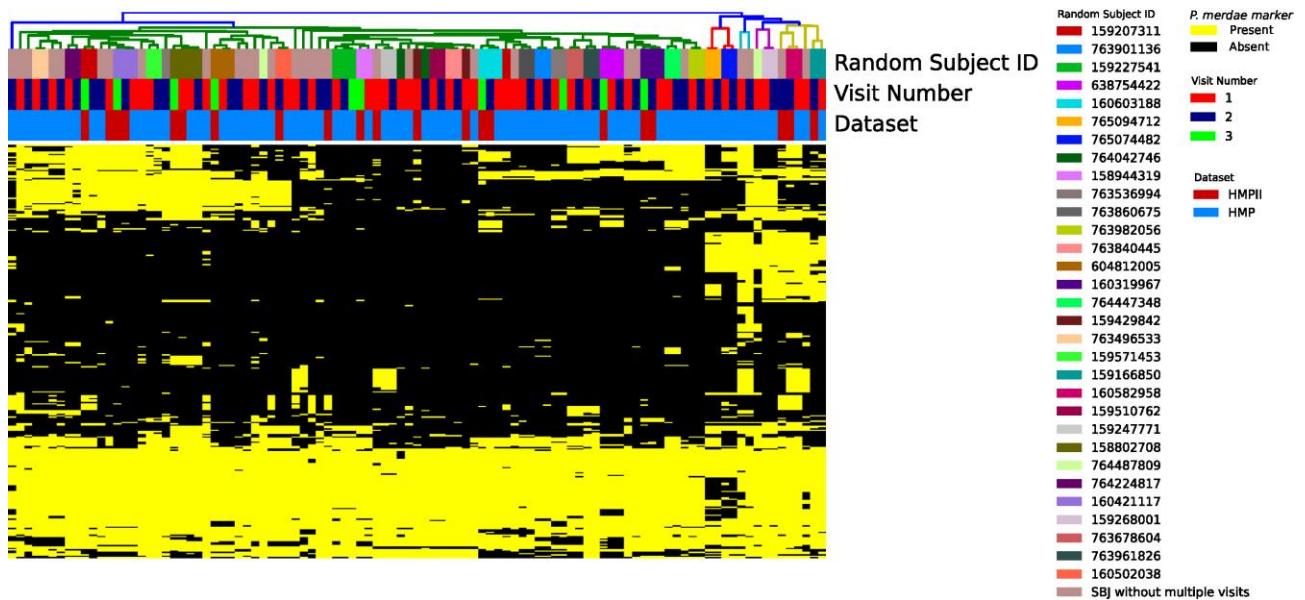
Supplementary Fig. 12. Strain level fingerprinting of *Prevotella copri* in HMP/HMP-II gut samples at multiple time points. The clustering step was performed based on Hamming distance



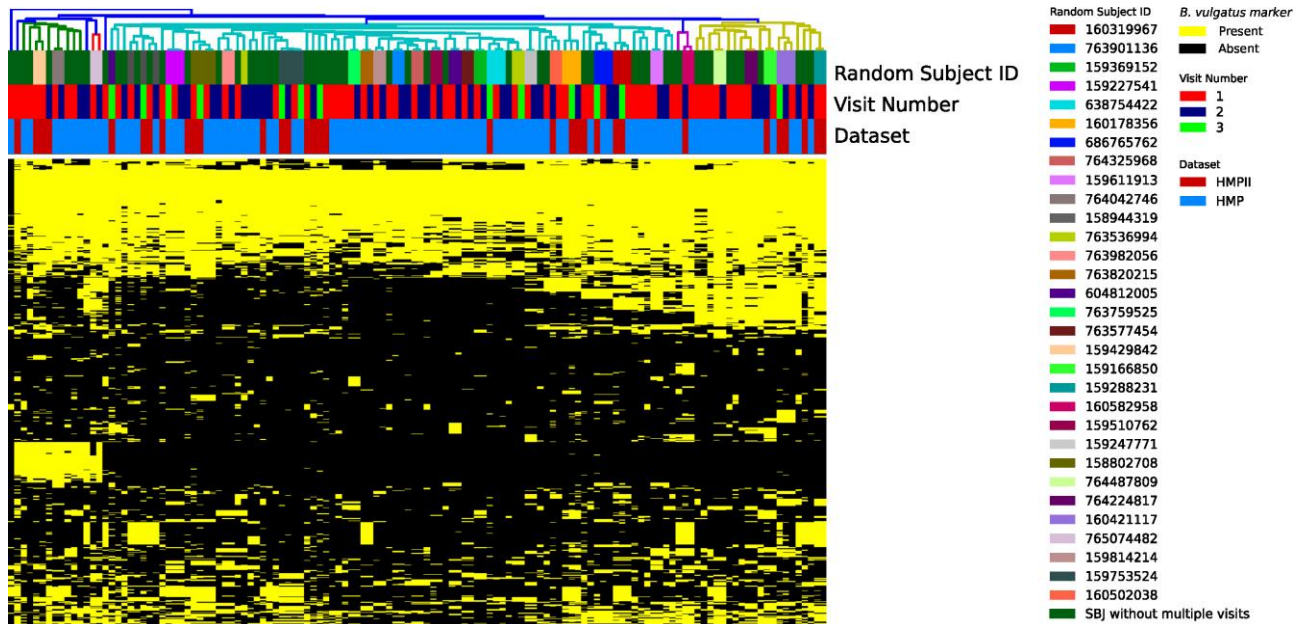
Supplementary Fig. 13. Strain level fingerprinting of *Alistipes putredinis* in HMP/HMP-II gut samples at multiple time points. The clustering step was performed based on Hamming distance



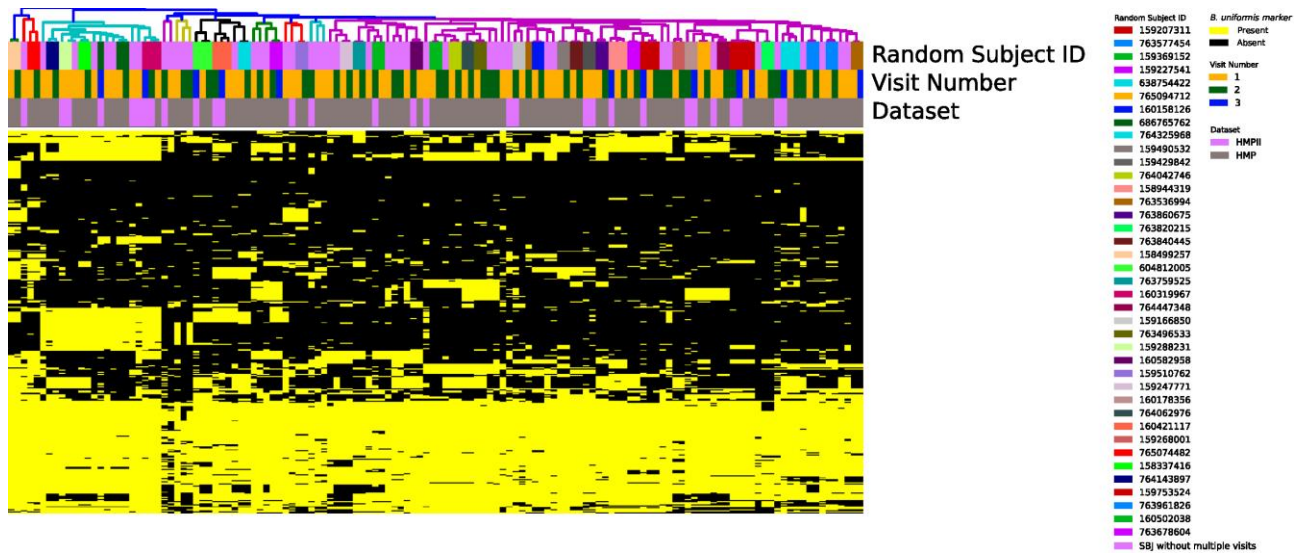
Supplementary Fig. 14. Strain level fingerprinting of *Eubacterium rectale* in HMP/HMP11 gut samples at multiple time points. The clustering step was performed based on Hamming distance



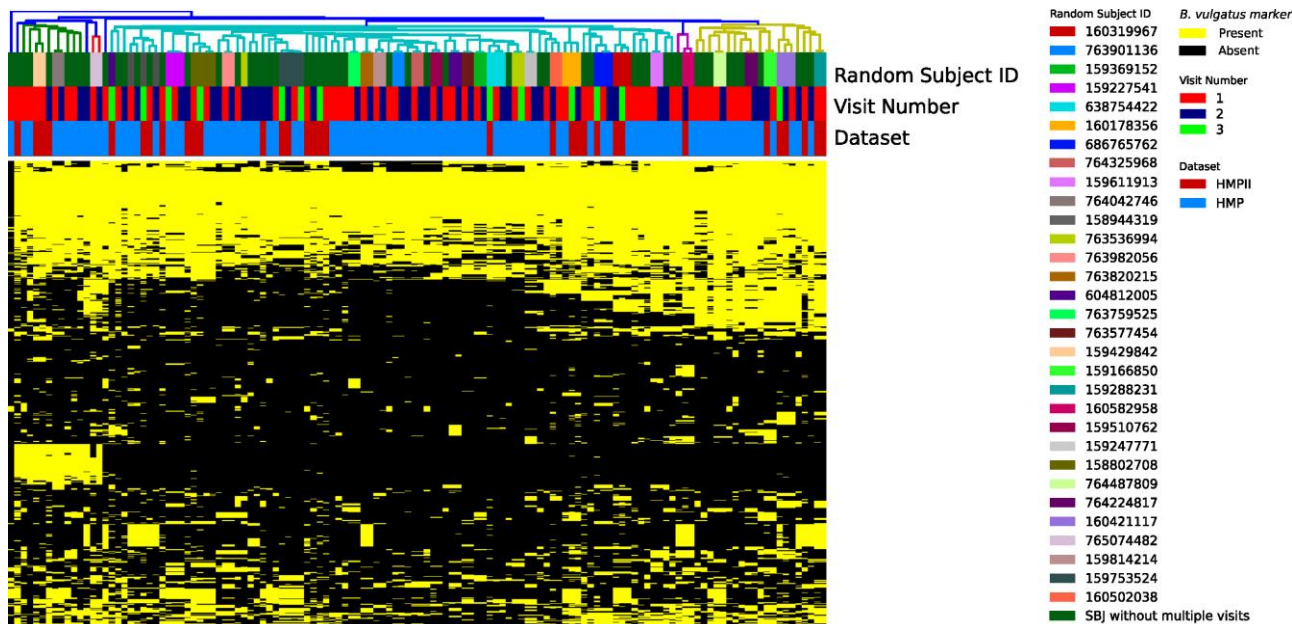
Supplementary Fig. 15. Strain level fingerprinting of *Parabacteroides merdae* in HMP/HMP11 gut samples at multiple time points. The clustering step was performed based on Hamming distance



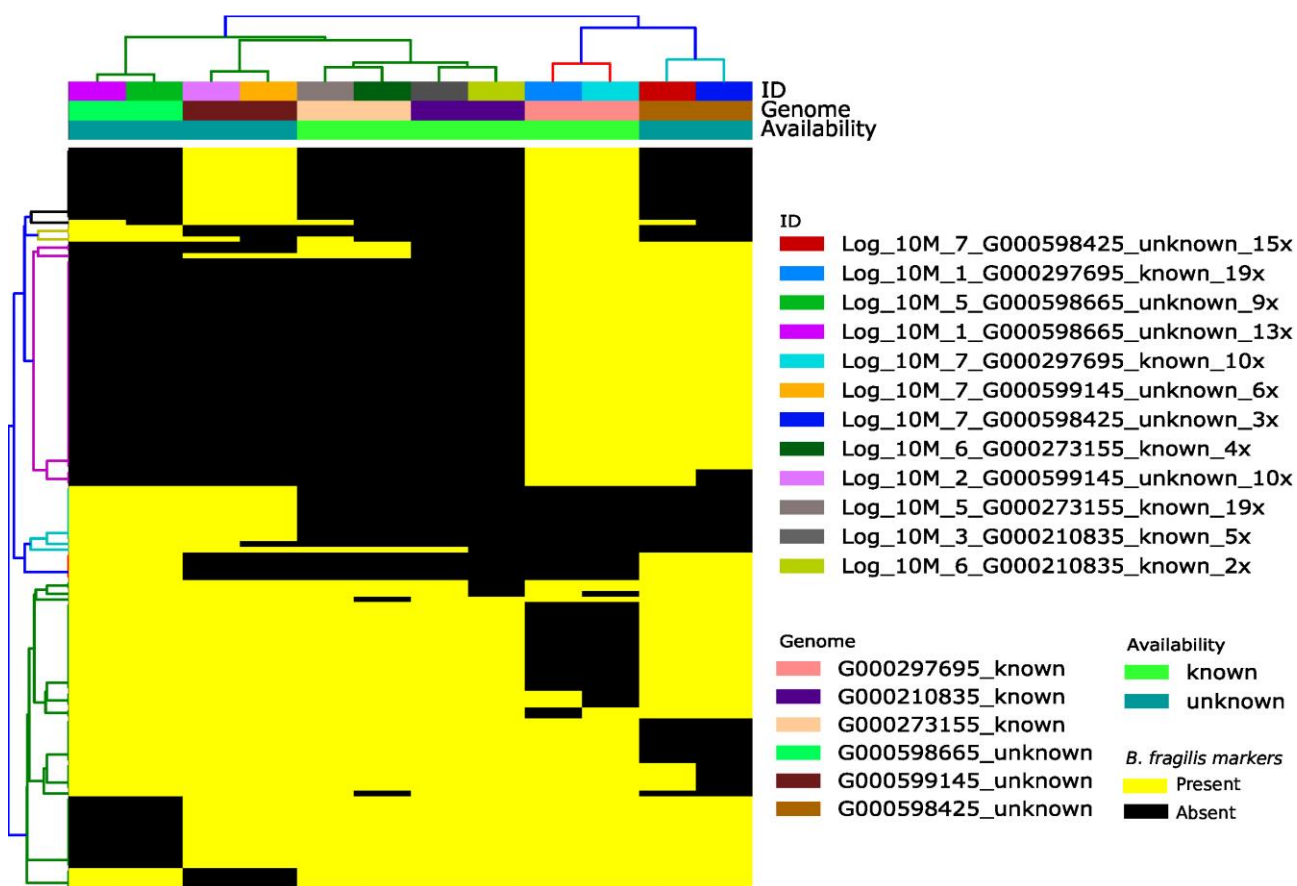
Supplementary Fig. 16. Strain level fingerprinting of *Bacteroides ovatus* in HMP/HMP-II gut samples at multiple time points. The clustering step was performed based on Hamming distance



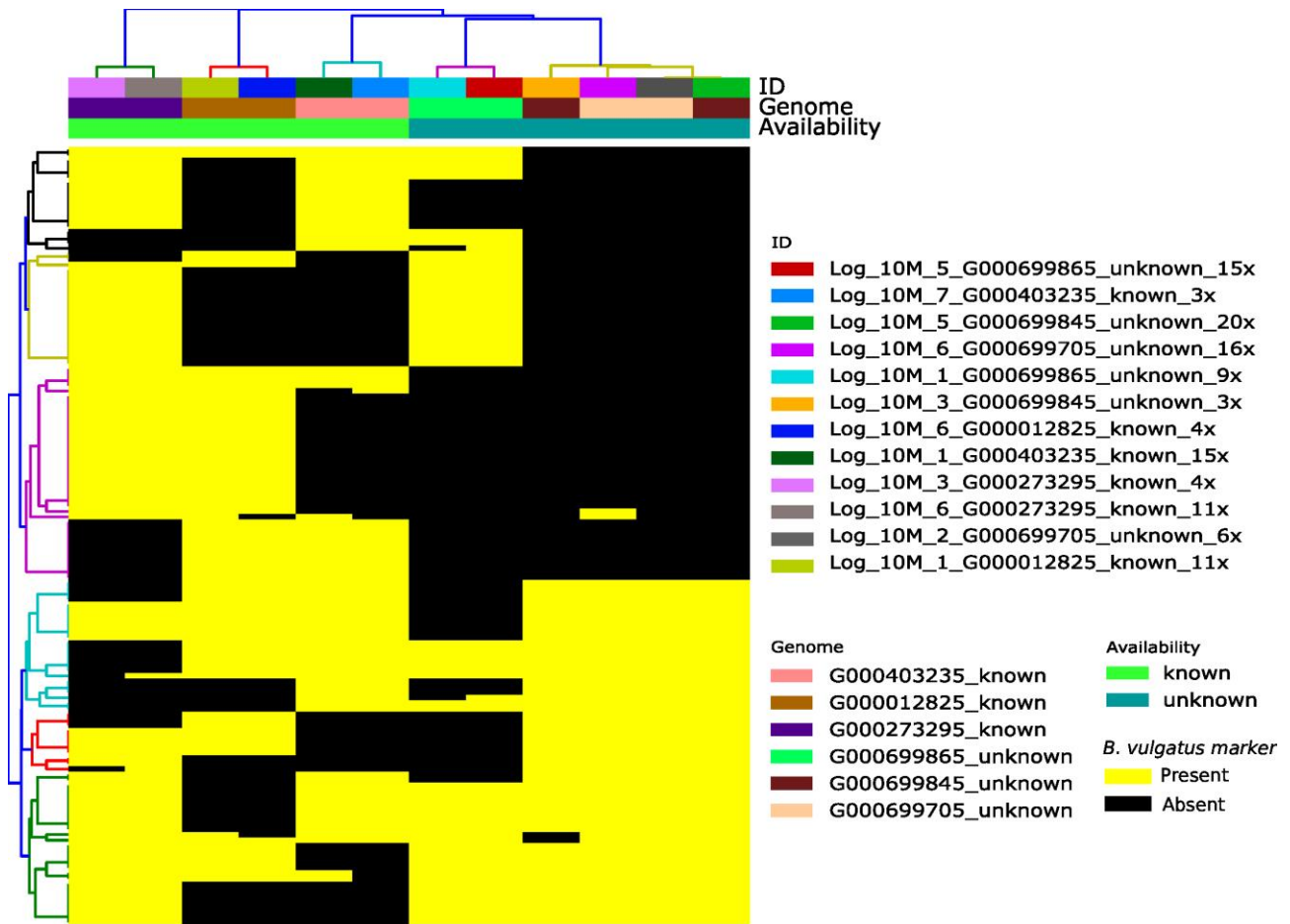
Supplementary Fig. 17. Strain level fingerprinting of *Bacteroides uniformis* in HMP/HMP-II gut samples at multiple time points. The clustering step was performed based on Hamming distance



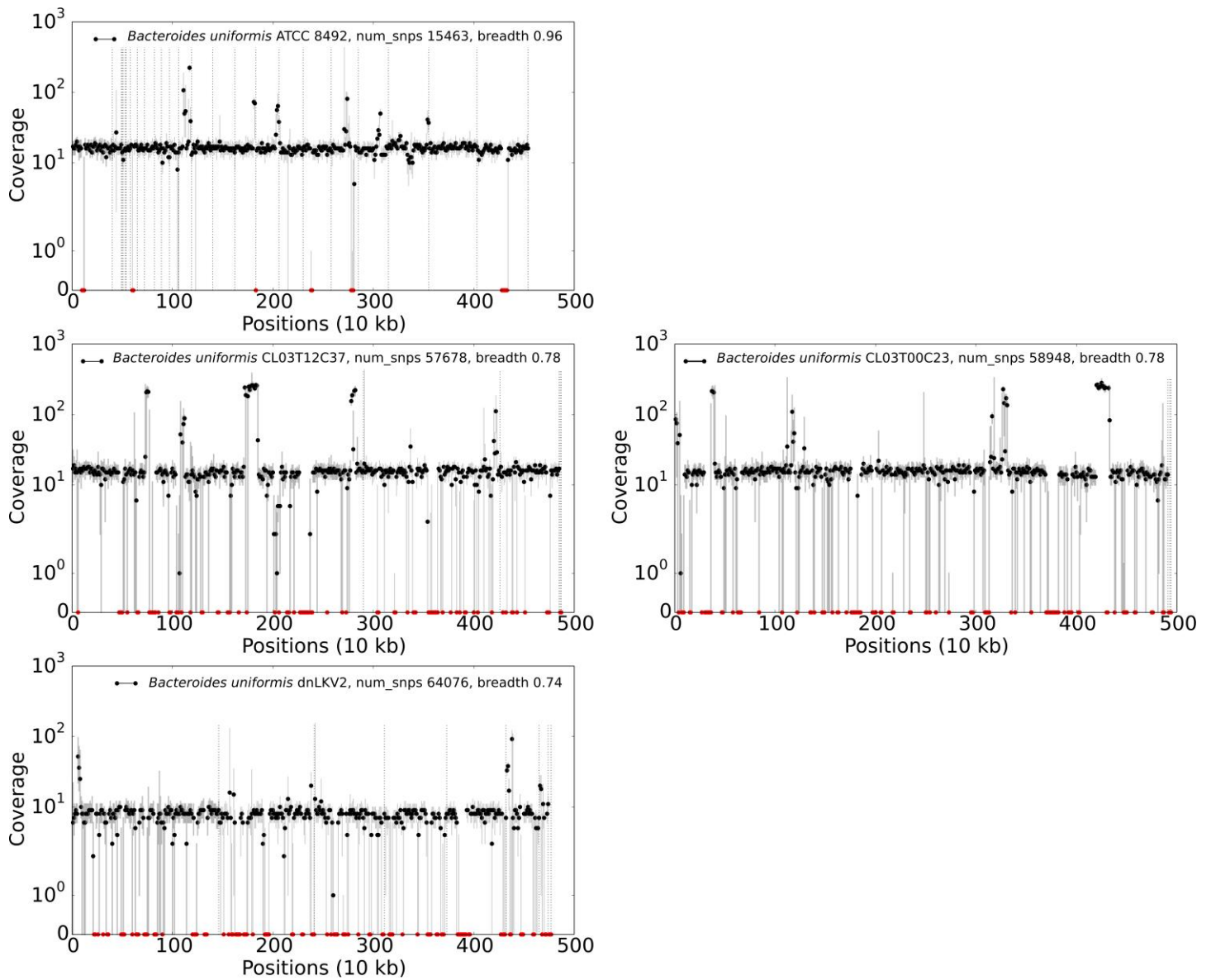
Supplementary Fig. 18. Strain level fingerprinting of *Bacteroides vulgatus* in HMP/HMP2 gut samples at multiple time points. The clustering step was performed based on Hamming distance



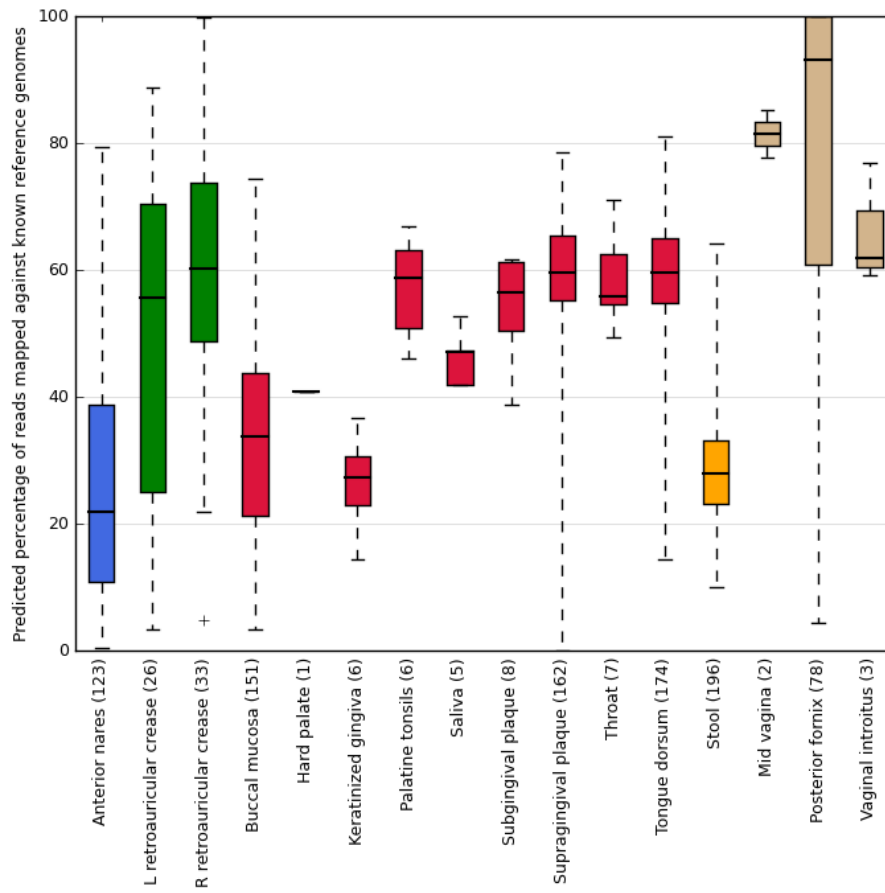
Supplementary Fig. 19. Strain-level fingerprinting of *Bacteroides fragilis* in twelve synthetic samples generated from its six genomes sampled at different coverage (3 unknown and 3 known genomes) and merged with different synthetic metagenomes



Supplementary Fig. 20. Strain level fingerprinting of *Bacteroides vulgatus* in twelve synthetic samples generated from its six genomes sampled at different coverage (3 unknown and 3 known genomes) and merged with different synthetic metagenomes



Supplementary Fig. 21. An example of strain identification for *Bacteroides uniformis* strains. The top left panel shows the genome coverage of the strain *Bacteroides uniformis* ATCC 8492 detected by MetaPhlan2 while the remaining panels depict the coverages of the other sequenced *Bacteroides uniformis* strains. Windows of 10kb with zero coverage are highlighted in red



Supplementary Fig. 22. Predicted percentage of reads mapped against known reference genomes for the HMP/HMP-II samples.

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