NEW RESULTS

Addressing the dynamic nature of reference data: a new nt database for robust metagenomic classification

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Abstract

Background
Accurate metagenomic classification relies on comprehensive, up-to-date, and validated reference databases. While the NCBI BLAST Nucleotide (nt) database, encompassing a vast collection of sequences from all domains of life, represents an invaluable resource, its massive size —currently exceeding 10^{12} nucleotides— and exponential growth pose significant challenges for researchers seeking to maintain current nt-based indices for metagenomic classification. Recognizing that no nt-based indices exist for the widely used Centrifuge classifier, and the last public version was released in 2018, we addressed this critical gap.
Results
We present a new Centrifuge-compatible nt database, meticulously constructed using a novel pipeline incorporating different quality control measures, including reference decontamination and filtering. These measures demonstrably reduce spurious classifications, and through temporal comparisons, we reveal how this approach minimizes inconsistencies in taxonomic assignments stemming from asynchronous updates between public sequence and taxonomy databases. These discrepancies are particularly evident in taxa such as *Listeria monocytogenes* and *Naegleria fowleri*, where classification accuracy varied significantly across database versions.
Conclusions
This new database, made available as a pre-built Centrifuge index, responds to the need for an open, robust, nt-based pipeline for taxonomic classification in metagenomics. Applications such as environmental metagenomics, forensics, and clinical metagenomics, require comprehensive taxonomic coverage and will benefit from this resource. Our new nt-based index highlights the importance of treating reference databases as dynamic entities, subject to ongoing quality control and validation akin to software development best practices. This dynamic update approach is crucial for ensuring the accuracy and reliability of metagenomic analysis, especially as databases continue to expand in size and complexity.

Key words: Metagenomics; Taxonomic Classification; Reference Database; NCBI BLAST nt; Centrifuge; Recentrifuge; Quality Control; Data Contamination; High Performance Computing

Background

The capacity to accurately and sensitively profile microbial communities is critical to a broad swath of biological applications, spanning biomedical research, healthcare, and environmental biosurveillance. Microbial community features are indicative of a multitude of human health-relevant outcomes [1], including cancer [2], infection [3, 4, 5], trauma [6, 7], chronic injury [8, 9], and neurological disease [10, 11, 12]. Similarly, profiling microbial communities in non-human environments can provide detection and association

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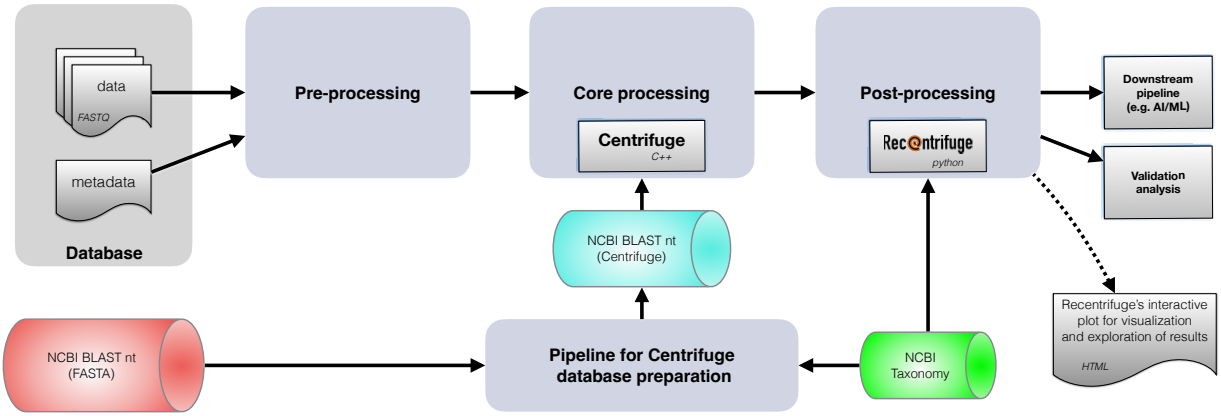

Figure 1. General layout of a metagenomic classification pipeline based on the NCBI BLAST nt database using Centrifuge and Recentrifuge. This flowchart is showing a typical configuration of a pipeline for taxonomic classification in metagenomics using the NCBI BLAST nt database [37, 39]. Data (and metadata, as available) is feed into a FASTQ format pre-processing block that performs quality checks using software such as fastp [40] and MultiQC [41]. Filtered FASTQ files are then processed by Centrifuge [20] using an indexed version of the nt database. Finally, Recentrifuge [36] post-process Centrifuge output, including negative control samples (if any), to further filter and prepare results for interactive exploration, downstream analysis and validation tests. Typical downstream jobs include metagenome assemblers and artificial intelligence/machine learning (AI/ML) training pipelines. The pipeline for building the Centrifuge reference database based on nt is detailed in Figure 3.

information relevant to bioremediation, environmental surveillance [13, 14], pest control [15], and wastewater monitoring [16, 17]. In these applications spaces, the microbial community is often profiled via metagenomic sequencing, which facilitates comprehensive detection of fastidious or difficult-to-culture microorganisms. Metagenomic classification transforms raw shotgun DNA sequences into microbiome composition profiles, which can then be interrogated for signatures or biomarkers of any given label of interest.

A broad array of software platforms have been developed for classification of shotgun metagenomic sequence reads, including Kraken [18, 19], Centrifuge [20], MetaPhlAn [21], CLARK [22, 23], DIAMOND [24], Kaiju [25], GOTTCHA [26], metakallisto [27], KMCP [28], LMAT [29, 30], and ganon [31]. Each of these platforms relies, in some form, on a reference database containing known sequences or sequence signatures from a subset of reference genomes. These databases may include a range of indexed reference genomes, a subset of discriminative k-mers (e.g., CLARK [22] and KrakenUniq [32]), or a curated set of marker genes (e.g., MetaPhlAn4 [21]). The selection of database and software platform has a clear and systematic impact on the resultant microbial profile, influencing downstream assessments [33]. Numerous additional benchmarking studies providing comparative assessments between platforms are available for assessment [34]. In addition to the reference source and taxonomic clades for inclusion, metagenomic classification results are impacted by temporal distribution of build dates and filtering processes applied to the reference content. These parameters are potentially impactful but have not been thoroughly assessed in previous studies.

To address this knowledge gap, we undertook a comparison of four temporally distinct database builds, comparing the extent of read classification and overall compositional impact. We also assessed the impact of reference decontamination via the Conterminator [35] and Recentrifuge [36] platforms and the impact of short reference sequence removal. For broad taxonomic coverage, we constructed databases from the NCBI BLAST Nucleotide (nt) database [37], the most comprehensive database for nucleotide BLAST search [38]. We selected the Centrifuge classifier platform for this comparison due to its classification speed and optimized indexing scheme.

The NCBI BLAST nt database encompasses nearly all traditional GenBank divisions, representing a significant portion of available GenBank sequences and spanning all domains of life [37, 39]. Therefore, the growth rate of nt follows that of GenBank. Both the number of sequences and the nucleotides in GenBank are experiencing an exponential growth (see Figure 2 for quantitative details). Building nt-based reference databases for taxonomic annotation requires increasing computational resources, such as processing power, memory, and I/O bandwidth. In particular, the build process for these databases is memory-intensive, requiring computing architectures that are not broadly available, with the literature mentioning initial database build difficulties and subsequent build failures as the dataset sizes increase[31, 42]. The latest available Centrifuge database based on NCBI BLAST nt was built in 2018, with multiple users unsuccessfully requesting an updated version in recent years [43]. As a response to the need for an open, robust, nt-based pipeline for taxonomic classification in metagenomics, we provide an updated, stable, and optimized Centrifuge nt reference database.

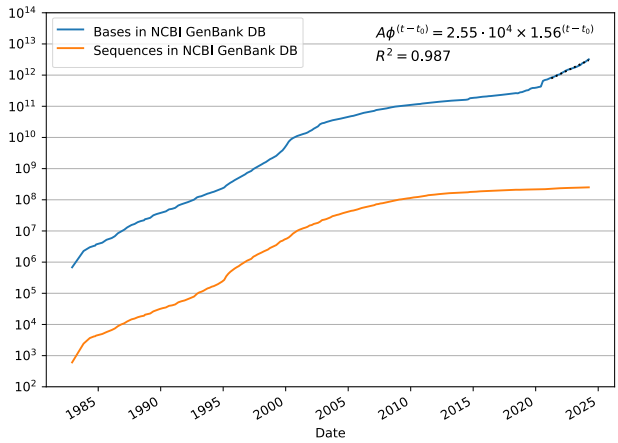

Figure 2. Evolution of NCBI GenBank database over time. This semi-log plot shows the number of bases and sequences for NCBI GenBank. For the last 3 years of the time series, from April 2021 to April 2024, we fit an exponential model $A\phi^{(t-t_0)/\tau}$ (with time constant $\tau = 1$) to the number of bases in the database, with R^2 confirming the goodness of the model. As time t (x-axis, date) is measured in years from $t_0 = 1982$, A is the intercept to $t = t_0$ and ϕ is the base of the exponential with the meaning of the yearly rate of change [44]. So, on average, between the mentioned dates, GenBank database had a 56% growing rate in nucleotides from year to year. In addition, at least in the last decade, the slope of the number of bases has been increasing while the slope of sequences has been decreasing, with these opposite trends indicating a progressive rise in the average length of the sequences in the database.

The updated database provides a critical resource for researchers

Table 1. Size parameters for nt DB references and builds

nt DB release date	Build version name	Masked nt DB size		Centrifuge nt DB size		Taxonomy DB	
		std	**dec**	**std**	**dec**	size	content
Jun 26, 2022	Jun22	-	745 GiB	-	442 GiB	203 MiB	2.43 Mtid
Sep 23, 2022	Sep22	-	843 GiB	-	500 GiB	213 MiB	2.45 Mtid
Jan 04, 2023	Jan23	941 GiB	936 GiB	558 GiB	555 GiB	217 MiB	2.48 Mtid
Apr 03, 2023	Apr23	1.09 TiB	1.08 TiB	615 GiB	611 GiB	220 MiB	2.50 Mtid

About database sizes, **dec** is a decontaminated database while **std** is a standard database (non-decontaminated).
The size of taxonomy database is the decompressed size in megabytes of the *names.dmp* file of the corresponding NCBI Taxonomy database available at the indicated nt database release date.
The unit tid denotes (different) taxonomic identifiers, so Mtid is a million of taxonomic identifiers.

using metagenomics, where comprehensive and reliable taxonomic classification across the tree of life is paramount. This work underscores the critical need for treating the databases as a dynamic entity requiring continuous quality control and validation, much like software development best practices.

Methods

Reference selection

Four NCBI nt release dates spanning 2022-2023 were selected for assessment (Table 1).

Characterization and decontamination of the reference database

Figure 3 shows a flowchart of the main steps involved in the pipeline for building a reference database for Centrifuge based on the nt database.

After obtaining the nt database in FASTA format using BLAST+ tools [45] and the current content of the NCBI Taxonomy database, the content of nt was explored using Recentrifuge [36]. Figure S1 shows some examples of insights into the database provided at this step.

The nt database was decontaminated using Conterminator [35] in parallel and the output post-processed by a different script of Recentrifuge, which removes the detected contamination and generated a contamination network plot (see Figure S2 for an example).

Reference masking, filtering, and indexing

The `refasplit` script from Recentrifuge [36] was used at different stages of the pipeline to enable efficient I/O processing of the sequence database in parallel, which contains nucleotide counts on the order of 10^{12}. Low complexity regions were masked using several instances of NCBI dustmasker [45] in parallel. The decontaminated and masked version of the nt database was then processed by Recentrifuge's `refafilt` code to remove sequences shorter than 16 nucleotides (found to cause problems downstream) and demultiplex headers for redundant sequences as generated by recent versions of the BLAST+ command line tool `blastdbcmd`. Finally, indexing of the decontaminated filtered database was performed with an optimized version of the Centrifuge 1.0.4-β database building C++ code `centrifuge-build` [20] targeting Livermore Computing (LC) HPC cluster nodes with 128 cores and 2 TiB main memory. The entire build process required about three weeks of clock time.

Sample sequencing and processing

Defined DNA mixtures were employed for comparative assessment of databases and classification parameters (Tables 1, 2). No-template process negative controls (ntc) were included in all analyses. Sequence libraries were prepared from gDNA via the Illumina DNA Prep library preparation kit and sequenced on the Illumina NextSeq 2000 via P2 300 cycle reagents and flow cell. Resultant data were processed for taxonomic classification via Centrifuge using each of the database builds described in Table 1. Comparative analyses of reference controls were carried out in R (version 4.2.0). Phyloseq [46] was used to generate microbiome abundance profiles from count data and ggplot2 [47] was used to generate figures. The vegan package [48] was used to compute Bray Curtis dissimilarity

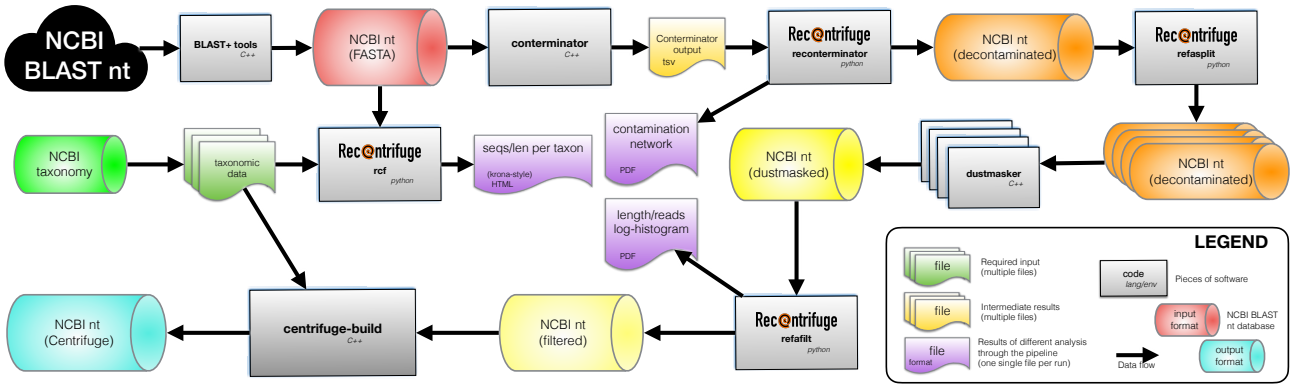

Figure 3. General flowchart of the pipeline for building the Centrifuge reference database based on the NCBI BLAST nt database. The entire pipeline process takes several weeks using a HPC (high-performance computing) server with a high-memory-per-core ratio, a high-bandwidth low-latency parallel filesystem, 128 cores, and total memory of 2 TiB. The most demanding step is the final indexing of the filtered database using a version of `centrifuge-build`, which may expand beyond two weeks, followed by the decontamination step, which usually takes a few days.

Table 2. DNA standard mixtures employed for comparative assessment

Sample name	Mixture	Vendor	Cat No	Human spike	Sample type
zymo_gut_microbiome	Zymobiomics gut microbiome	Zymo	D6331	no	bacteria
zymo_micro_comm_II	Zymobiomics microbial community standard II	Zymo	D6311	no	bacteria
atcc_mycobiome	Mycobiome mock community	ATCC	MSA-1010	no	eukaryote
atcc_oral_spike	Oral microbiome mock community	ATCC	MSA-1004	18 ng	bacteria
atcc_skin_spike	Skin microbiome mock community	ATCC	MSA-1005	14 ng	bacteria
atcc_gut_spike	Gut microbiome mock community	ATCC	MSA-1006	40 ng	bacteria
protozoa_mix	*Acanthamoeba castellanii, Naegleria fowleri*	ATCC	Various	no	eukaryote
mammal_equal_mix	Equal mix of 11 mammalian DNAs	Various	Various	no	eukaryote
non-mammal_mix	Equal mix of 10 non-mammalian eukaryotic DNAs	Various	Various	no	eukaryote
mammal_log_mix	Log concentration range of 7 mammalian DNAs	Various	Various	no	eukaryote
atcc_20_strain	ATCC 20 strain mix	ATCC	MSA-1003	no	bacteria
atcc_path_mix	ATCC pathogen mix	ATCC	MSA-4000	no	bacteria

distances between samples.

Results and Discussion

Comparative assessment of gDNA reference controls and mixtures

In addition to the assessment of temporal database builds, we evaluated other microbiome analytical parameters relevant to the assessment of accurate microbiome profiles. This warrants the use of standard controls with known microbial composition across different kingdoms of life (Tables 1, 2). We systematically evaluated the impact of different values for the minimum length of partial hits in Centrifuge [20] or MHL (Minimum Hit Length), relative abundance thresholds, taxonomic levels, and sample type to generate accurate microbiome profiles. We calculated precision, recall, balanced F1 scores and Bray Curtis dissimilarity distances to compare sequenced profiles of control standards with ground truth data across all database versions (Table 2).

First, we investigated the impact of temporal database builds on microbiome profiling and observed temporal instability in Bray Curtis dissimilarity distances in 3 samples; ATCC_path_mix, protozoa_mix and zymo_micro_comm_II when compared to reference control profiles (Figures 4, S3). There was an increase in Bray Curtis dissimilarity distances in the Jan23 database versions for these samples. Upon inspection, we determined that delayed updates between taxonomic labels in NCBI BLAST nt database and the current NCBI Taxonomy database at the time of the nt database release was a major factor. The nt database is relying on the Taxonomy database for the taxonomic information, but both databases are evolving over time independently, with no explicitly version mapping between them in the documentation. Traditionally, the nt database was using a current version of the taxonomy database but that does not seem the case anymore, with the taxonomy used in NCBI BLAST nt database slightly trailing behind. In some cases, newly uploaded genomic sequences were lacking current taxonomic labels and then confounded Centrifuge's database indexing algorithm and, eventually, its classification process.

For example, in the protozoa_mix sample, there were reads that were assigned and equally scored to multiple *Naegleria fowleri* (tid 5763) reference genomes that were present in the Jan23 NCBI BLAST nt database. However, when Centrifuge was reporting multiple assignments (Centrifuge default), only reads that were assigned to *N. fowleri* genomes whose tid was present in the NCBI Taxonomy database used during the database compilation were assigned to tid 5763 while reads that were assigned to *N. fowleri* genomes whose tid was absent from the used taxonomy (but present in the NCBI BLAST nt database) were classified with a tid 0, normally reserved for unclassified reads. When Centrifuge was configured to report a unique tid per read using the lowest-common-ancestor (LCA) strategy (pipeline default), those reads were given a final root-only assignment (tid 1). As another example, the results for the zymo_micro_comm_II sample were impacted when using the Apr23 database due to varied taxonomic classification of *Listeria monocytogenes* genomes (Figures 4B, S3). In that databse, the addition of a *Listeria monocytogenes* genome (CP046449.1) annotated as Listeria sp. LM90SB2 (tid 2678528 in the NCBI Taxonomy database, with parent being unclassified *Listeria*, tid 2642072) resulted in a final LCA classification of reads at only the genus level (*Listeria*). Overall, our observations highlight the impact on the classification of specific taxa due to the discrepancy of taxonomic information between the nt database and the version of the NCBI Taxonomy database used for indexing the Centrifuge database.

Next, we tested the impact of the following MHL values: 15 (minimum value), 22 (Centrifuge's default), 30, 40, and 50 on the number of unique NCBI tax ids assigned to sequenced reads. Here, we observed that the number of unique NCBI tax id assignments and Bray Curtis dissimilarity distances decreases at increasing MHL values (Figure 4A,B). An inflection point was observed at MHL 30 (Figure 4A), followed by an increase in F1 and precision scores compared to MHL 15 and 22 (Figure 4D). This suggests that a MHL of 30 should be close to the optimal value in reducing false positive assignments across these particular samples (Figure S3B) by reducing the noise of spurious assignments without a significant increase in false negatives.

As most microbiome analysis protocols remove low-abundance features to account for potential sequencing errors and false positives, we assessed the impacts of different thresholds (0, 0.001, 0.01, 0.1, 0.5, 1) on classification performance. Overall, we observed that a relative abundance threshold of 0.1% resulted in the highest F1 scores across samples (Figure 4D). As expected and previously observed, our data demonstrated that classification performance decreases at lower taxonomic ranks with an increasing number of detected false positives (Fig. S3B). Furthermore, samples consisting mainly of bacterial content had a better classification performance than eukaryotic samples (Figure 4C,D).

Finally, we compared the number of annotated sequences between the different versions of the Jan23 database and observed a decrease along the implemented improvements (Fig 4C). The standard version (Jan23_std) had an average number of 26,309,720 annotated sequences, followed by the decontaminated version (Jan23_dec) with 26,298,931 annotated sequences and lastly, the decontaminated and filtered version (Jan23_filt) had 26,219,236 annotated sequences. However, Jan23_dec and Jan23_filt databases had higher non-root to root-only assignment ratios, suggesting that additional annotated reads with the standard version (Jan23_std) were assigned to the root-only level.

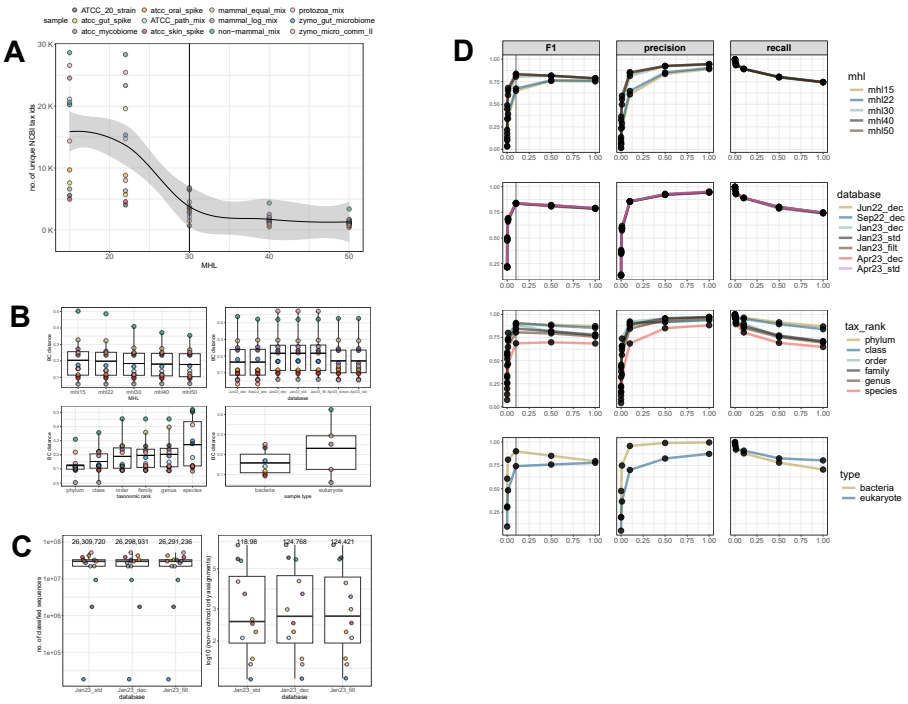

Figure 4. Comparative assessment of gDNA reference controls. A) The number of unique NCBI tid captured at different MHL values across samples and averaged across all temporal database versions. Data points are colored according to sample. B) Comparison of Bray Curtis dissimilarity distances at an abundance threshold of 0.1% between MHL values, database versions, taxonomic ranks, and sample types. For every variable that is being compared, Bray Curits dissimilarity distances were averaged across other variables. C) The number of annotated sequences and ratio of non-root to root-only assigned reads across different versions of the Jan23 database. Mean values are annotated at the top of each boxplot. D) Comparison of classification accuracy metrics (F1 balanced accuracy, precision, and recall) between MHL values, database versions, taxonomic ranks, and sample types across abundance thresholds.

Potential implications

While this work primarily focuses on providing a readily usable and improved nt-based database for metagenomic classification with Centrifuge and Recentrifuge, the underlying methodology and findings have implications that extend beyond this immediate application.

Firstly, the availability of a comprehensive, decontaminated nt database opens new avenues for developing and training machine learning models for metagenomic analysis. Such models, trained on cleaner data, have the potential to achieve higher accuracy and better generalization capabilities, particularly for challenging tasks such as identifying novel or rare taxa. This could lead to improved tools for metagenome-based diagnostics, discovery of novel microbial enzymes, and understanding the complex interactions within microbial communities.

Secondly, our observation of temporal inconsistencies in taxonomic assignments stemming from asynchronous updates between NCBI databases highlights a crucial challenge in metagenomic analysis. It underscores the need for a paradigm shift in how we approach reference data, moving from static resources to dynamically maintained and versioned collections. Drawing inspiration from software development best practices, continuous integration and continuous delivery (CI/CD) pipelines could be adapted for reference databases, ensuring timely updates, robust validation, and improved reproducibility across studies.

Additionally, the pipeline developed for database construction, encompassing decontamination, filtering, and validation steps, can be generalized to other large reference databases commonly employed in metagenomic analysis. Applying similar quality control measures to resources like the NCBI nr (non-redundant protein) database or specialized databases for viral or fungal identification could significantly enhance the accuracy and reliability of classification in those domains. This has the potential to improve research outcomes in fields as diverse as environmental monitoring, disease diagnostics, and evolutionary biology.

Furthermore, the availability of a comprehensive, up-to-date, and validated database encompassing all domains of life—such as the one presented here—offers a unique advantage for researchers in fields that require analysis of complex microbial communities across diverse environments. For example, studies investigating host-microbe interactions, exploring the microbiome of underexplored ecosystems, or tracing the origins of emerging pathogens can benefit greatly from such a resource.

However, the exponential growth of sequencing data and the associated computational burden highlight the need for innovative strategies to ensure the long-term sustainability of such comprehensive resources. This might involve exploring distributed computing approaches, developing more efficient indexing and search algorithms, splitting the database, or even dividing the classification problem, e.g., converting the classification goal in a hierarchical problem by using a "forest" of specific classifiers to decide the annotation at different taxonomic levels instead of a single classifier covering the entire tree of life.

Data availability

As of May 2024, the Jan23 decontaminated and filtered Centrifuge nt database is the updated database version. It can be downloaded from LLNL Green Data Oasis (GDO) public ftp server at `ftp://gdo-bioinformatics.ucllnl.org/centrifuge/nt_wntr23` using any software supporting anonymous ftp download. To ease the download process, the database is split in 71 ultra-compressed `7z` files of

4 GiB or less with name format `nt_wntr23_filt.cf.7z.*`. The corresponding taxonomy files for that version of the database, useful for Recentrifuge runs, are available at ftp://gdo-bioinformatics.ucllnl.org/centrifuge/nt_wntr23/taxonomy.

Shotgun metagenomic sequences of standard controls are uploaded to NCBI SRA with the Bioproject ID PRJNA1118346.

Practical notes

Despite the most demanding step is the indexing of the nt database as described above, even running the Centrifuge classifier with such a large indexed database has some important computational requirements, especially regarding free disk space and available main memory. The size of the ultra-compressed database files amount to 284 GiB. Once decompressed from this format, the size of the Centrifuge (compressed) database files is 422 GiB. Given that size, a computer with at least half TiB of main memory is recommended for running the Centrifuge classifier, especially when using a high number of parallel threads. In addition, Centrifuge may take more than half an hour just for loading the nt database from disk into memory. Given this overhead, it is recommended to use a sample sheet file (`-sample-sheet` argument in Centrifuge) to process multiple samples without requiring additional database loads. Also, when replicating our pipeline, it is strongly recommended to use the LCA strategy with Centrifuge, by using the `-k 1` argument in the call to the classifier. Finally, it is straightforward to use Recentrifuge to post-process Centrifuge's results, with detailed documentation in Recentrifuge's wiki at https://github.com/khyox/recentrifuge/wiki/Running-recentrifuge-for-Centrifuge.

Declarations

List of abbreviations

DB: Data Base; HPC: High Performance Computing; I/O: Input/Output; LCA: Lowest Common Ancestor; MHL: Minimum Hit Length; WGS: Whole-Genome Shotgun;

Competing Interests

The authors declare that they have no competing interests.

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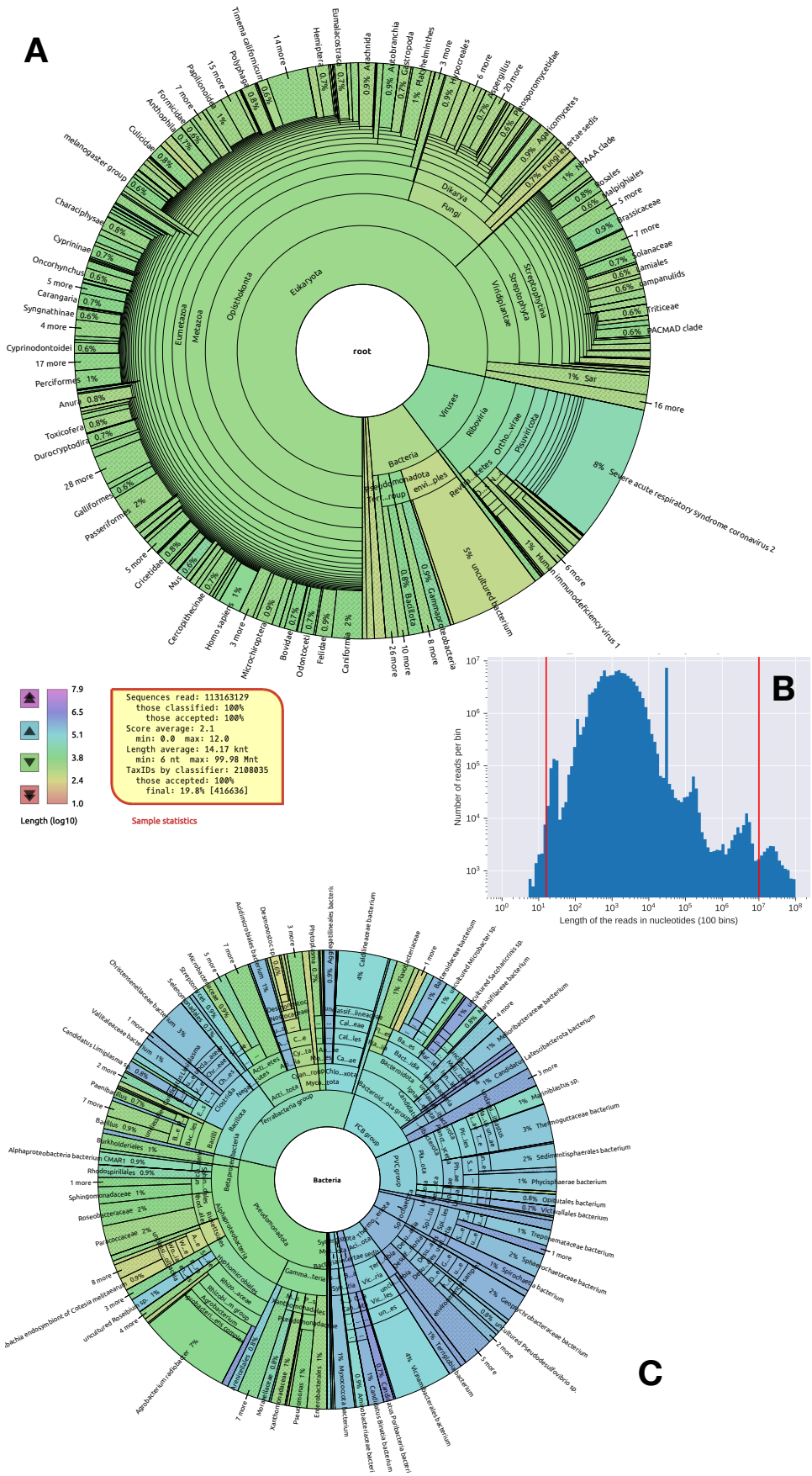
A
root
Eukaryota
Opisthokonta
Metazoa
Eumetazoa
Dikarya
Fungi
Viridiplantae
Streptophyta
Streptophyta
Viruses
Riboviria
Bacteria
Sar
16 more
Severe acute respiratory syndrome coronavirus 2
Human immunodeficiency virus 1
uncultured bacterium
Cyprinodontoidei
17 more
Perciformes
Anura
Toxicofera
Durocryptodira
28 more
Galliformes
Passeriformes
Criceti...
Muroidea
Cercopithecinae
Homo sapiens
Microchiroptera
Bovidae
Odontoceti
Felidae
Caniformia
Bacillota
Gammaproteobacteria
Sequences read: 113163129
those classified: 100%
those accepted: 100%
Score average: 2.1
min: 0.0 max: 12.0
Length average: 14.17 knt
min: 6 nt max: 99.98 Mnt
TaxIDs by classifier: 2108035
those accepted: 100%
final: 19.8% [416636]
Sample statistics
Length (log10)
7.9
6.5
5.1
3.8
2.4
1.0
B
Number of reads per bin
Length of the reads in nucleotides (100 bins)
C
Bacteria
Terrabacteria group
FCB group
PVC group
Pseudomonadota
Gammaproteobacteria
Alphaproteobacteria
Betaproteobacteria
Bacillota
Clostridia
Bacilli
Bacteroidota
Cyanobacteriota
Actinomycetota
Spirochaetota
Planctomycetota
Verrucomicrobiota
Thermotogota
Thermoguttaceae bacterium
Sedimentisphaerales bacterium
Phycisphaerae bacterium
Opitutales bacterium
Victivallales bacterium
Treponemataceae bacterium
Spirochaetaceae bacterium
Geobacter sp.
Nitrosomonadaceae bacterium
uncultured Pseudodesulfovibrio sp.
Ureaplasma parvum
Ureaplasma urealyticum
Mycoplasma hominis
Mycoplasma fermentans
Mycoplasmatales bacterium
Candidatus Bilanema bacterium
Candidatus Frankiella bacterium
Candidatus Bilanema bacterium
Candidatus Frankiella bacterium
Candidatus Limnoplasma sp.
Candidatus Limnoplasma endosymbiont of Cotesia melitaearum
Candidatus Hodgkinia cicadicola endosymbiont of Cicadella viridis
Candidatus Hodgkinia cicadicola endosymbiont of Dryocosmus kuriphilus
Candidatus Hodgkinia cicadicola endosymbiont of Tettigoniella ferrugineoguttata

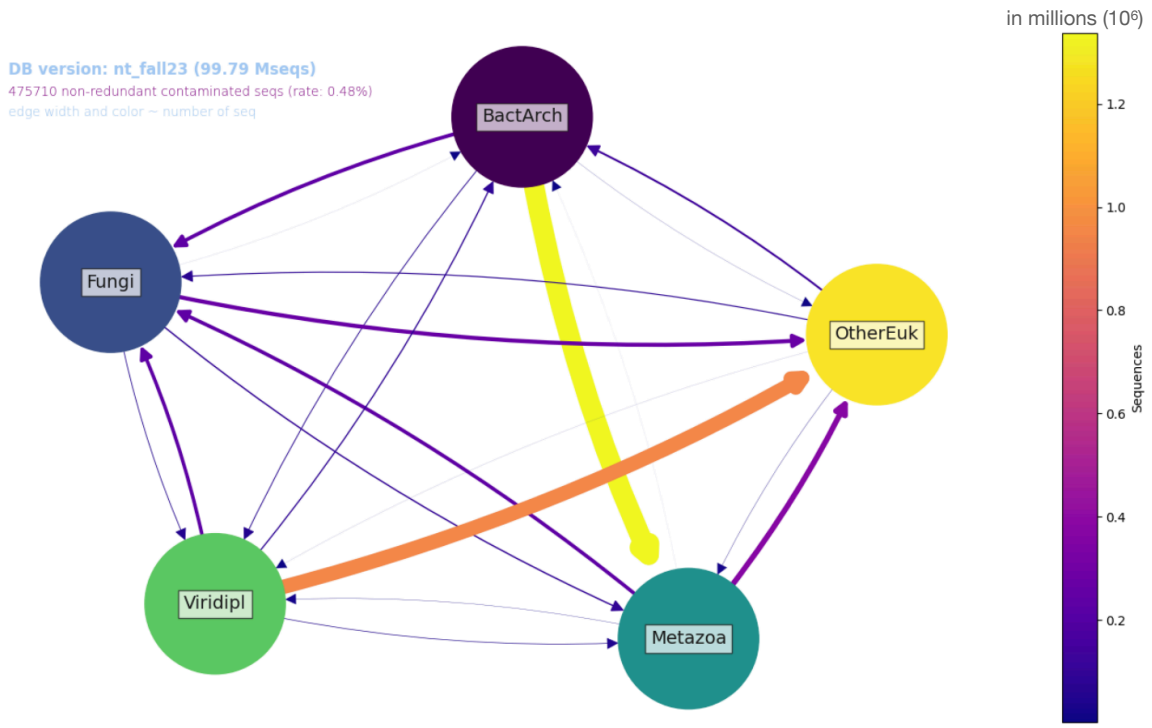

Figure S2. Example of inter-kingdom contamination network plot generated during the process of building the nt database for Centrifuge. The contamination was detected between the five default "kingdoms" as defined in Conterminator software, with the width and color scale of the arrows related to the number of sequences involved. The rate of contamination detected in the database (non-redundant sequences) before contamination removal was significant: almost half a percentage point.

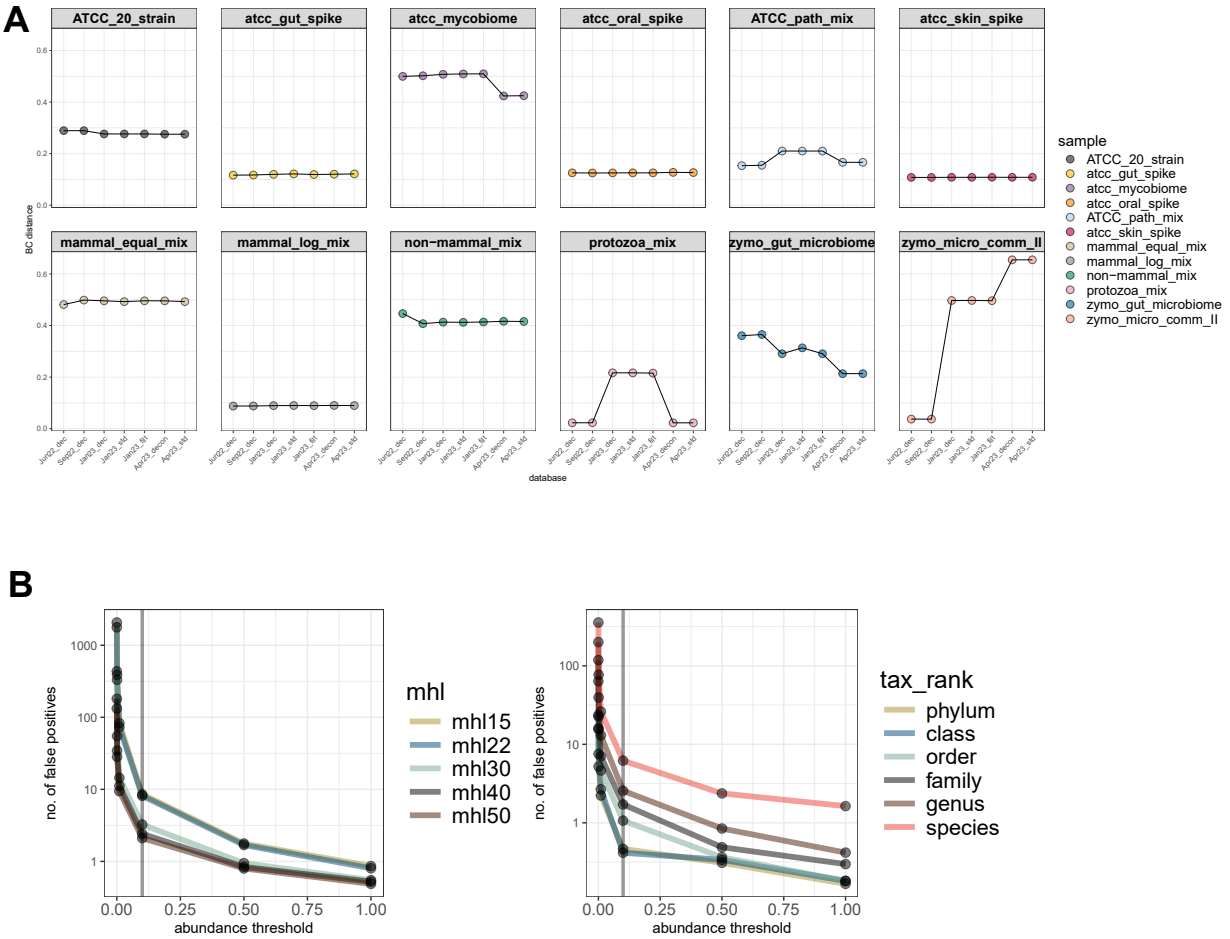

Figure S3. Additional comparative assessment of gDNA reference controls. A) Bray Curtis dissimilarity distance between database versions across all gDNA reference control samples. B) Number of false positive assignments between MHL values and taxonomic rank across abundance thresholds.