Improved eukaryotic detection compatible with large-scale automated analysis of metagenomes

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Abstract

Background

Eukaryotes such as fungi and protists frequently accompany bacteria and archaea in microbial communities. Unfortunately, their presence is difficult to study with shotgun sequencing techniques, since prokaryotic signals dominate in most environments. Recent methods have made detection of known eukaryotes possible using eukaryote-specific marker genes, yet they do not incorporate strategies to handle presence of unknown eukaryotes.

Results

Here we present CORALE (for Clustering of Reference Alignments), a tool for identification of eukaryotes in shotgun metagenomic data that uses the previously reported EukDetect marker gene reference, but increases sensitivity of detection through multiple alignments and Markov clustering. Using a combination of simulated datasets and large publicly available human microbiome studies, we demonstrate that our method not only improves sensitivity, but is also capable of inferring the presence of eukaryotes not included in the marker gene reference, such as novel species and strains. We then deploy CORALE on our MicrobiomeDB.org resource, demonstrating adequate reliability and throughput.

Conclusion

CORALE allows eukaryotic detection to be automated and carried out at scale. Since our approach is independent of the reference used, it is applicable to other contexts where shotgun metagenomic reads are matched against redundant but non-exhaustive databases, like identification of novel bacterial strains or taxonomic classification of viral reads.

Background

Eukaryotic microbes are a large and phylogenetically diverse group of organisms that includes both pathogens and commensals, the latter of which are emerging as important modulators of health and disease. Protists include many important pathogens of humans and other animals, such as *Cryptosporidium*, *Toxoplasma*, *Eimeria*, *Trypanosoma*, and *Plasmodium*. Many fungi are also well-studied pathogens affecting a diverse range of hosts. For example, *Aspergillus fumigatus* is an important cause of respiratory disease in humans ("Aspergillus Fumigatus and Aspergillosis" 1999); *Magnaporthe oryzae* is the most important fungal disease of rice globally (Wilson and Talbot 2009); while *Pseudogymnoascus destructans* is the cause of White-Nose Syndrome, one of the most devastating diseases of bats (Wibbelt et al. 2010). However, recent data also suggests that non-pathogenic commensal fungi are significant as modulators of the human antibody repertoire (Doron, Leonardi, et al. 2021),(Doron, Mesko, et al. 2021),(Ost et al. 2021); intestinal barrier integrity, and colonization resistance (Leonardi et al. 2022), (Jiang et al. 2017). This diverse array of host-microbe interactions and host phenotypes influenced by eukaryotic microbes underscores the importance of studying this class of organisms in their natural habitats. Unfortunately, the ability to carry out culture-independent analysis of eukaryotic microbes is severely hindered by their low abundance relative to bacteria, which makes accurate detection a challenge and means that these organisms are commonly overlooked in metagenomic studies (Laforest-Lapointe and Arrieta 2018). For example, an analysis of stool metagenomes in healthy adults participating in the Human Microbiome Project (Consortium et al. 2012) shows only 0.01% reads aligning to fungal genomes (Nash et al. 2017).

Several methods have been developed to improve the detection of eukaryotes in complex samples. Targeted sequencing of internal transcribed spacer regions (ITS) (Schoch et al. 2012) is a common approach but prevents simultaneous profiling of other members of the microbiome. Alternatively, collections of curated fungal genomes have been successfully used for strain-level identification of Blastocystis from stool (Beghini et al. 2017), however, pitfalls associated with non-specific or erroneous parts of reference genomes (R Marcelino, Holmes, and Sorrell 2020) together with computational challenges associated with carrying out alignments to very large collections of reference genomes (Burrows and Wheeler 1994), (Breitwieser, Baker, and Salzberg 2018), mean that alternative approaches are needed to enhance the discovery of eukaryotes from the vast amount of metagenomic data already available in the public domain. One attractive solution to this challenge was recently proposed in important work by Lind and Pollard (Lind and Pollard 2021), who base their method for sensitive and specific identification of eukaryotes in metagenomic studies, EukDetect, on alignments to over 500,000 universal, single-copy eukaryotic marker genes.

We recently sought to add EukDetect results our web-based resource, MicrobiomeDB.org (Oliveira et al. 2018), in order to allow eukaryote detection across

a range of human metagenomic studies currently available on the site. Since the EukDetect pipeline does not allow for adjustment of filtering thresholds and it is not packaged for containerised deployments, we decided to implement our own tool, with a more flexible software architecture. We retained EukDetect's reference of marker genes with the aim of producing directly comparable results, kept `bowtie2` (Langmead and Salzberg 2012) since it has been shown to be a sensitive aligner (Thankaswamy-Kosalai, Sen, and Nookaew 2017), and conducted a simulation of reads from marker genes to better understand the filtering process used by EukDetect. We noticed that a filter based on MAPQ scores, whose function is to remove uncertain hits, also removes good alignments. Studying MAPQ scores in simulated data has shown us that alignments to marker genes reflect relations of each source of reads to parts of the reference that are most similar to it. This led us to develop CORALE (for Clustering of Reference Alignments), an approach to processing marker gene alignments based on exploiting information in shared alignments to reference genes through clustering. CORALE is able to sensitively detect eukaryotes from the reference while also enabling inference of novel species not present in the reference.

Results

Species-specific impact of MAPQ filtering

We base our study of alignments to marker genes on the `wgsim` (Li 2011) software for sampling reads, `bowtie2` used with EukDetect's settings for aligning the reads we sample, and a custom Python program for processing the results: we capture properties of alignments like the MAPQ score (Li et al. 2009) together with degree of mismatch between source taxon of each read and the taxon of the sequence it aligns to. To quantify outcomes, we calculate the proportion of sampled reads that correctly map (recall), as well as the correctly mapped reads as a proportion of total reads that map to any reference (precision) (Meyer et al. 2019).

Not surprisingly, when reads in a metagenomic sample are simulated from the reference and then aligned back, thus exactly matching the reference, they are accurately mapped to the correct taxon with a precision and recall of 95.1%. Applying a MAPQ >= 30 filter increases precision to 99.7% and decreases recall to 91.7%. This translates to 8% of reads mapping with MAPQ < 30 with just under half of those being incorrectly mapped. In contrast, with MAPQ >= 30 only 0.3% are incorrectly mapped.

Stratifying these values by the source taxon of the reads reveals a structural component to the difficulty of mapping the reads, as well as the efficacy of the MAPQ >= 30 filter (Figure 1). For example, out of 3977 taxa whose reads map back to the reference, reads from 1908 map with 100% precision (Figure 1, red points), and after applying the MAPQ >= 30 filter, 1105 more taxa map with 100% precision. Despite this clear improvement after applying the MAPQ filter, 146 taxa still map with precision lower than the pre-filter overall total of 95.1% (Figure 1, dotted line). This includes numerous species of Aspergillus (Figure 1A), Leishmania (Figure 1B), and Trichinella (Figure 1C), all of which are important pathogens of humans and other mammals. Furthermore, applying the MAPQ filter results in decreased precision for five taxa, including the fungi *Fusarium cf. fujikuroi NRRL 66890* and *Escovopsis sp. Ae733*, as well as the protists *Favella ehrenbergii*, *Leishmania peruviana*, and *Mesodinium rubrum*.

Since the diversity of eukaryotic taxa far exceeds the currently discovered species, let alone species present in the EukDetect reference (Mora et al. 2011), we then modify this experiment to study the possibility of detecting 'novel' species: we split the species level markers in the EukDetect reference into a hold-out set of 371 taxa and a reference set of 3343 remaining taxa, sample from the hold-out set, and align to an index built from the reference set. In these circumstances, the MAPQ >= 30 filter is not on average an improvement: same-genus precision and recall are 82% and 30% without the filter, comparing to 83.6% precision and a much-diminished recall of 7% with the filter. Source taxon is a structural component here, too: while applying the MAPQ >= 30 filter increases the number of taxa which only map to the correct genus from 48 to 152, it also

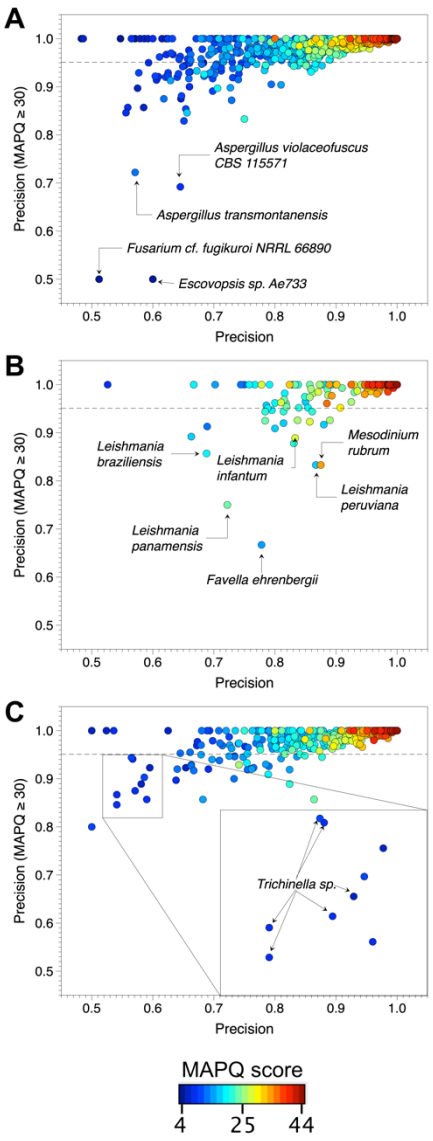

Figure 1: **The improvement from the MAPQ >= 30 filter varies by species.**

increases the number of taxa which don't map at all from 49 to 175.

We also study the possiblity of 'somewhat novel' species - like a non-reference strain of a known species - with the `wgsim` mutation rate model. We start from aligning non-modified reads back to the reference as in the first experiment, and increase the mutation rate in increments, which makes the alignment problem increasingly harder. Over the [0, 0.200) range of `wgsim` mutation rate parameter M, recall declines from 95.1% to below 10% while precision stays between 95-96% for all reads and >= 99% for reads with MAPQ >= 30, consistently with bowtie2 preserving precision over recall [16]. Applying the MAPQ >= 30 filter causes recall to drop much faster: for example, when M = 0.1, recall is 68.3% without the MAPQ >= 30 filter and 5.0% with the filter.

CORALE leverages Markov clustering for reference-based eukaryote detection

w We wrote our software, CORALE - for Clustering of Reference Alignments - as a Nextflow workflow wrapping a Python module. It provisions sequence files, aligns them to the reference of markers, and produces a taxonomic profile through a seven-step procedure (Figure 2). First, we run `bowtie2` and keep all alignments that are at least 60 nucleotides in length (Figure 2, step 1), which ensures that the matches contain enough information to be marker-specific. We then run Markov Clustering (MCL) on a graph of marker genes as nodes and counts of shared alignments as edge weights (Figure 2, step 2) to obtain marker clusters. We then calculate % match identities of alignments (Figure 2, step 3) and aggregate them by marker to obtain an identity average for each marker gene, as well as per cluster to obtain a cluster average. Each marker whose identity average is lower than the cluster average is an inferior representation for signal in the sample, so we next reject each taxon with >= 50% of such markers (Figure 2, step 4). We then group remaining taxa into taxon clusters using MCL with counts of multiply aligned reads (Figure 2, step 5), which allows us to reflect ambiguity of identification in reporting the hits. We then report unambiguous matches (defined as having average alignment identity of at least 97%, two different reads aligned to at least two markers) as is (Figure 2, step 6), while rejecting other taxa in taxon clusters where there were any unambiguous matches reported. Finally, for each remaining taxon cluster, we report it as one hit if is a strong ambiguous match (defined as having at least four markers and eight reads) by joining names of taxa in the cluster and prepending with a "?" (Figure 2, step 7).

A CORALE user can alter this procedure or adjust any thresholds. We set it as default based on our observations in simulated and human microbiome data, but the software also supports a few other filters developed in the course of our experimentation, like a filter based on the fraction of primary to secondary alignments for each taxon, which is a simpler but slightly inferior alternative to the filter based on marker cluster averages. Additionally, CORALE has rich reporting capabilities - for example, we produce 'copies per million (CPMs)', a quantitative estimate of abundance calculated as number of reads normalized by marker length and sequencing depth, and its flexible software architecture is based on a SQLite database which allows for custom reporting and easy addition of new filters.

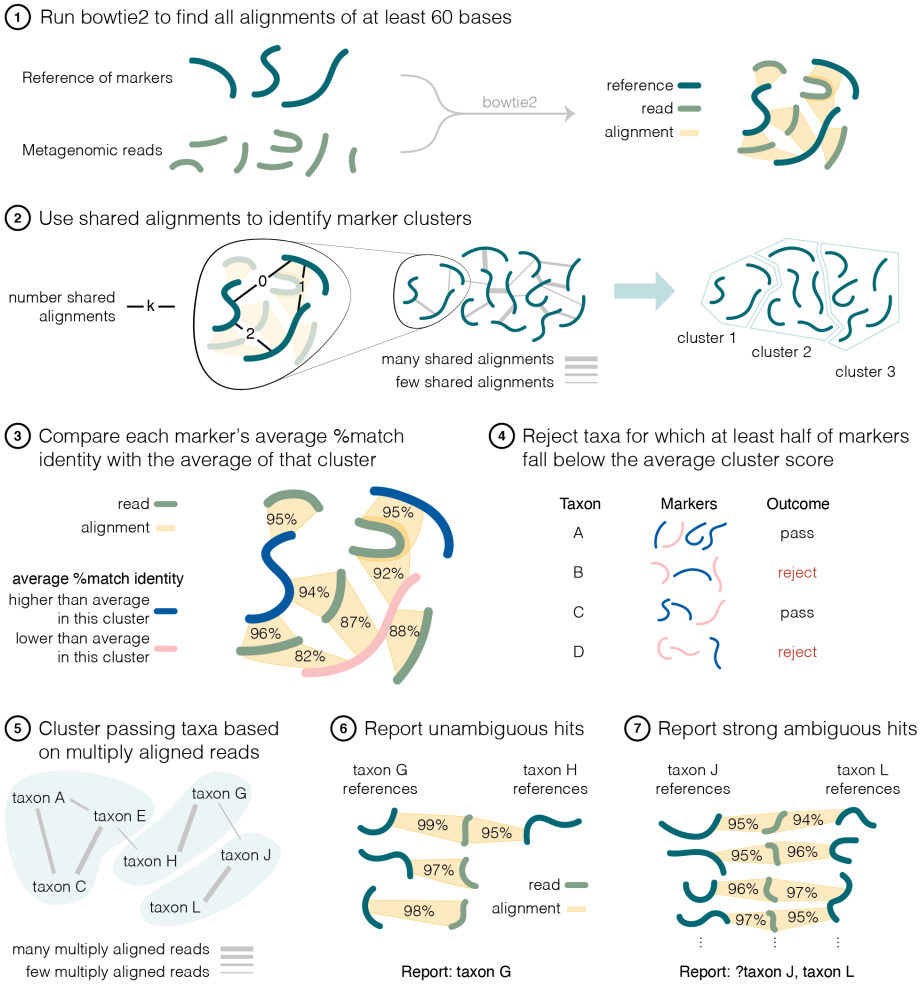

Figure 2: **CORALE - schematic**

CORALE is capable of inferring presence of novel species

To demonstrate CORALE's ability to report sensible results when the provided reference does not contain a species producing a signal, we return to the hold-out set and remaining reference described before, and prepare an input of 338 samples each with a single 'novel' eukaryotic species at 0.1 genome coverage. We compare CORALE, EukDetect, "EukDetect+": a version of EukDetect modified to filter MAPQ >= 5 instead of MAPQ >= 30, and "EukDetect-": a run of `bowtie2` like in EukDetect followed by reporting taxa where least four reads align with MAPQ >= 30 to at least two markers. We judge correctness measured by taxonomic proximity - whether results are the same genus as the 'novel' source of reads - as well as correctness measured by correct plurality of results.

CORALE does best at reporting one result in the correct genus (Figure 3). The MAPQ >= 30 filter makes inferring novel species more difficult, since EukDetect's proportion of No Hits is higher than for EukDetect+ and exactly the same as EukDetect-. Modifying EukDetect to filter on MAPQ >= 5 is not an adequate adjustment, because while it improves the tool's ability to recognise eukaryotic signal in the sample, it compromises the tool's ability to recognise that this signal consists of only a single species. Our method maintains the second ability while improving the first one - fractions of samples where signal is detected for CORALE, EukDetect, EukDetect+, and EukDetect- are respectively 0.607, 0.346, 0.47, and 0.346, and fractions of detected signal reported as one species are respectively 0.8, 0.812, 0.604, and 0.632.

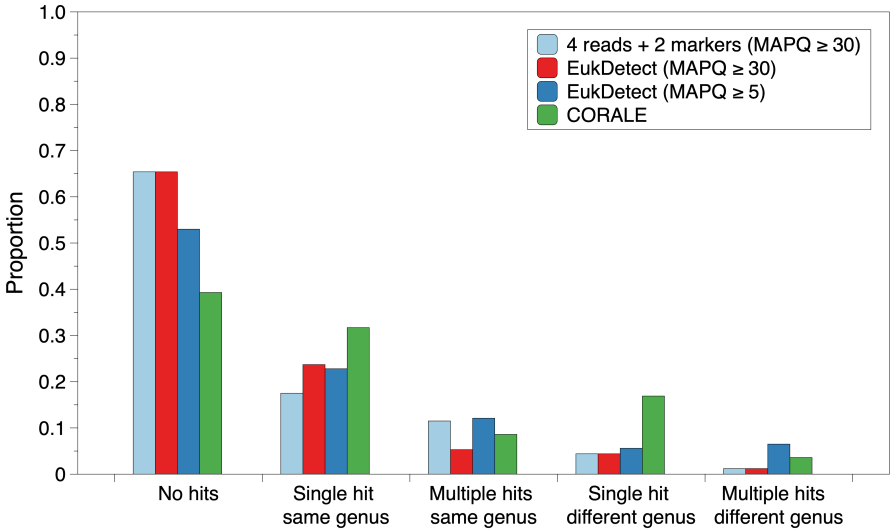

Figure 3: **Simulated unknown taxa - 0.1 coverage**

Evaluating CORALE on human microbiome data

To test our method on data where there are expectations about which eukaryotes might be present, we turn to the the DIABIMMUNE study (Vatanen et al. 2016), for which 136 data points about 30 different eukaryotes were reported across 1154 samples in the original EukDetect publication (Lind and Pollard 2021). Processing these same 1154 samples, CORALE is in exact concordance with EukDetect on 122/136 data points, and adds additional 97 data points. CORALE reports common taxa at a higher frequency, for example, *S. cerevisiae* appears 67 times, while EukDetect only identifies this organism 31 times, suggesting higher sensitivity. No additional hit reported by CORALE is obviously implausible - they consist primarily of yeast and other fungi that have been previously reported in the human gut - and the lack of absurd results that happen when alignments to taxon-specific databases are used without caution (R Marcelino, Holmes, and Sorrell 2020) confirms specificity is not compromised.

Importantly, CORALE differs from EukDetect in how it treats reads that might originate from a novel species. For example, in sample G78909 from DIABIMMUNE, EukDetect reports *Penicillium nordicum*, while our method reports a novel *Penicillium*. In sample G80329, our method agrees with EukDetect regarding detection of *Candida parapsilosis*, and also identifies an additional *C. albicans*. Finally, in sample G78500 EukDetect reports *Saccharomyces cerevisiae* and *Kazachstania unispora*, which our method reports to be reads from one taxon slightly different from the reference *Saccharomyces cerevisiae*.

Automating eukaryote detection with CORALE

In addition to making our software freely available, we integrate CORALE into the automated data loading workflow for our open-science platform, MicrobiomeDB.org. As of Release 25 (2 Dec 2021), the site contains 5113 samples from 6 published metagenomic studies Gasparrini et al. (2019), and CORALE identifies 97 distinct fungal species across 1661/5113 (32%) metagenomic samples. A summary of the top 10 most frequently observed fungi (Figure 4A) reveals that *Malassezia restricta*, a common commensal and opportunistic pathogen, and *Candida albicans*, a prevalent component of gut flora, are most commonly detected, with presence in 363 and 229 samples, respectively. Since these results are integrated with all other sample annotations on MicrobiomeDB, users can easily identify associations between eukaryotes and metadata (Figure 4B and 4C). For example, *Malassezia globosa* (identified in 130 samples) is primarily found on skin and nostrils, while *C. albicans* (Figure 4B and 4C), *C. parapsilosis*, *Clavispora lusitaniae*, and *S. cerevisiae* are all primarily or exclusively found in stool.

A

Top 10 fungal species detected

across all metagenomic studies on MicrobiomeDB

species	sample count
Malassezia restricta[1]	363
Candida albicans[2]	229
Purpureocillium lilacinum[3]	181
Saccharomyces cerevisiae[2]	145
Malassezia globosa[1]	130
Candida parapsilosis[2]	113
Clavispora lusitaniae[2]	60
Cyberlindnera jadinii	43
Candida tropicalis	32
Malassezia sympodialis[1]	17

[1] Primarily on skin and nostrils

[2] Primarily or exclusively in stool

[3] Exclusively in infant gut

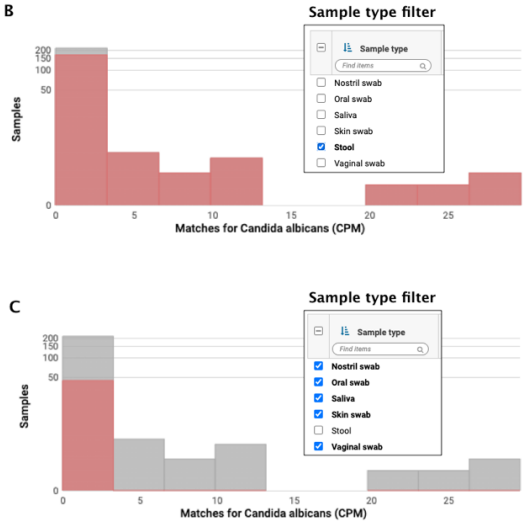

Figure 4: **MicrobiomeDB - top fungal species**

Discussion

Use cases and limitations of CORALE

CORALE is a tool with the same aim as EukDetect (Lind and Pollard 2021): to report on eukaryotes in metagenomes without overwhelming the results by false positives. Thanks to our novel approach, we achieve better sensitivity, similar specificity, and additional capabilities, and our tool is reliable enough to let us integrate eukaryotic detection into our open-science platform, MicrobiomeDB.org, and process thousands of samples. CORALE empowers the microbiome research community with a means of setting up broad screens of metagenomic data to identify samples where eukaryotes are present.

Unfortunately, the approach of using shotgun metagenomics followed by processing with CORALE is not without limitations, chiefly due to high cost of WGS sequencing to the depth required to detect most eukaryotes. CORALE handles these limitations gracefully, making use of minimal information required to plausibly report unambiguous hits. Future improvements in genome assembly could be of help here through yielding better information on eukaryote-specific genomic sequences which could be used to create a larger reference with more taxa and more sequences per taxon, but CORALE does not require a complete reference - it is able to infer presence of taxa which are absent from the reference.

Future work

Our approach could potentially be applied to processing alignments to any reference that is anticipated to be redundant and incomplete, and where reads are expected to map with varying identity. This includes identification of bacteria to the strain-level resolution required in genomic epidemiology, as well as taxonomic classification of viral reads.

Additionally, the efficacy of using many alignments per read in combination with clustering demonstrated by CORALE shows that to develop new metagenomics tools, it can be worthwhile to view protein sequences as a similarity-based network: naturally occuring proteins form isolated clusters of similar sequence (Smith 1970). With additional theoretical work, this view could become a basis for predictions about presence of eukaryotes, potentially providing probabilistic estimates of certainty on reported results.

Conclusion

CORALE (for Clustering of Reference Alignments) is a tool for identification of eukaryotes in shotgun metagenomic studies in which the results are not overwhelmed by false positives. While CORALE is based on the same marker gene reference as EukDetect, it does not use EukDetect's approach to filtering, most notably not including the MAPQ >= 30 filter which we show to have species-specific impact on results. Its approach, based on multiple alignments and Markov clustering, results in sensitive and accurate detection, and is capable of inferring presence of eukaryotes not included in the reference. We show this to be the case using simulated samples with 'novel' species, as well as data from DIABIMMUNE, a large infant gut metagenome study. CORALE is also successfully deployed on our MicrobiomeDB.org resource, demonstrating the appropriateness of our method for large-scale screens of metagenomic data for the purpose of detecting eukaryotes.

Methods

To conduct simulations, we use `wgsim` (Li 2011) to simulate reads from the 1/23/2021 version of EukDetect's reference, latest at time of writing, consisting of BUSCOs from OrthoDB (Kriventseva et al. 2019). We use `bowtie2` (Langmead and Salzberg 2012) to align reads to references, in end to end (default) mode and the `--no-discordant` flag as in EukDetect. When using `wgsim` we set read length to 100, and base error rate to 0.

To check correctness of simulated alignments, we retrieve the rank of the nearest taxon containing source and match by using the ETE toolkit (Huerta-Cepas, Serra, and Bork 2016) and the NCBI database version dated 2020/1/14 packaged with EukDetect. We deem the alignment correct if the source and match are of the same species, and in case of hold-out analysis where the species is missing from the reference by construction, same genus.

To simulate whole samples, we skip 33 inputs where `wgsim` considers too fragmented to source reads from at a set coverage, yielding 338 samples. We calculate the number of reads to source per marker to obtain 0.1 coverage as in (Sims et al. 2014).

To run EukDetect, we edit the default config file such that it lists the simulated samples. For the MAPQ >= 5 modification ("EukDetect+"), we additionally modify source code of our local installation. For the "four reads mapping with at MAPQ >= 30 to at least two markers" modification, ("EukDetect-"), we run CORALE in non-default configuration to use these three filters.

Data availability

All our software is publicly available under the MIT license: CORALE (*github.com/wbazant/CORALE*), its main Python module, (*github.com/wbazant/marker_alignments*), and a mix of Python, Make, and Bash scripts to produce simulations, comparisons, and figures for this publication (*github.com/wbazant/markerAlignmentsPaper*).

All results are publicly viewable and downloadable on MicrobiomeDB. In addition, the following files are available as supplemental material:

Simulated whole samples - results for different methods

Simulated reads - per-species breakdown and aggregate stats

DIABIMMUNE - CORALE vs EukDetect comparison

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