CCMetagen: comprehensive and accurate identification of eukaryotes and prokaryotes in metagenomic data

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Abstract

High-throughput sequencing of DNA and RNA from environmental and host-associated samples (metagenomics and metatranscriptomics) is a powerful tool to assess which organisms are present in a sample. Taxonomic identification software usually align individual short sequence reads to a reference database, sometimes containing taxa with complete genomes only. This is a challenging task given that different species can share identical sequence regions and complete genome sequences are only available for a fraction of organisms. A recently developed approach to map sequence reads to reference databases involves weighing all high scoring read-mappings to the data base as a whole to produce better-informed alignments. We used this novel concept in read mapping to develop a highly accurate metagenomic classification pipeline named CCMetagen. Using simulated fungal and bacterial metagenomes, we demonstrate that CCMetagen substantially outperforms other commonly used metagenome classifiers, attaining a 3 – 1580 fold increase in precision and a 2 – 922 fold increase in F1 scores for species-level classifications when compared to Kraken2, Centrifuge and KrakenUniq. CCMetagen is sufficiently fast and memory efficient to use the entire NCBI nucleotide collection (nt) as reference, enabling the assessment of species with incomplete genome sequence data from all biological kingdoms. Our pipeline efficiently produced a comprehensive overview of the microbiome of two biological data sets, including both eukaryotes and prokaryotes. CCMetagen is user-friendly and the results can be easily integrated into microbial community analysis software for streamlined and automated microbiome studies.

Introduction

Microbial communities in natural and host-associated environments commonly harbour a mix of bacteria, archaea, viruses and microbial eukaryotes. Bacterial diversity has been extensively studied with high-throughput sequencing (HTS) targeting 16S rDNA markers (Caporaso et al. 2011; Taberlet et al. 2012). However, these do not amplify eukaryotic sequences, and our knowledge on the diversity and distribution of microbial eukaryotes is limited (Bik et al. 2012; Norman et al. 2014). Although there is an increasing number of studies using eukaryotic-specific markers, these are relatively uncommon and face multiple methodological limitations (Piganeau et al. 2011; Marcelino and Verbruggen 2016). The problematic amplification step can be bypassed by sequencing the total DNA (metagenome) or RNA (metatranscriptome) in a sample to characterize all the genes contained or expressed within it. Metagenomics and metatranscriptomics are promising tools to bridge the knowledge gap in the diversity of microbial eukaryotes because they are essentially kingdom-agnostic, are less susceptible to amplification bias, and yield a large set of genes that can be used for taxonomic identification.

Multiple software packages have been developed to reveal the species composition of metagenomic samples (reviewed in Breitwieser et al. 2017). While well-known bacterial species can be easily identified at the species and strain levels (Truong et al. 2015; Scholz et al. 2016), it remains challenging to obtain a fine-grained taxonomic classification of lesser-known species and microbial eukaryotes (Sczyrba et al. 2017; Nilsson et al. 2019). Many of the current metagenomic classifiers assign a taxonomy to each short sequence read individually (Breitwieser et al. 2017). However, as closely-related species share very similar or identical genome portions, short reads often map to multiple species in the reference data set. Some metagenomic classifiers, like MEGAN (Huson et al. 2007) and Kraken (Wood and Salzberg 2016), address this issue by calculating the lowest common ancestor (LCA) among all species sharing those sequences. Paradoxically, this classification strategy is negatively affected by the increasing size of reference databases: as identical regions in reference databases become more common, fewer reads can be classified at the species level (Nasko et al. 2018). Other classifiers use a database of clade-specific diagnostic regions (e.g. Truong et al. 2015). While highly accurate, this procedure relies heavily on reference databases of complete genomes, which often cannot be readily updated by the end-user. Complete genomes are available for only a small fraction of the microbial eukaryotic species. For example, as of April 2019, the widely used

86 NCBI RefSeq database contained 285 fungal genome sequences, even though it is estimated that
87 there are over 2 million species of fungi (Hawksworth and Lucking 2017). Therefore, relying on these
88 databases of complete genomes restricts the inclusion of microbial eukaryotes in metagenome
89 studies.

90 A recently-developed concept in read mapping – the ConClave sorting scheme, implemented
91 in the KMA software (Clausen et al. 2018) – is more accurate than other mapping strategies as it
92 takes advantage of the information from all reads in the data set (Figure 1). Our goal was to use this
93 approach to produce an accurate metagenomic classification pipeline that will allow the inclusion of
94 microbial eukaryotes in metagenomic studies. We present a novel tool - CCMetagen (ConClave-
95 based Metagenomics) – to process KMA sequence alignments and produce highly accurate
96 taxonomic classifications from metagenomic data. We benchmark CCMetagen using simulated fungal
97 and bacterial metagenomes and metatranscriptomes. Additionally, we use two case-studies with real
98 biological data to demonstrate that CCMetagen effectively produces a comprehensive overview of the
99 eukaryotic and prokaryotic members of microbial communities.
100
101

102 ## Results

103 *Implementation and availability*

104 Metagenomic reads (or contigs) are first mapped against a reference database with KMA (Clausen et
105 al. 2018), which implements the ConClave sorting scheme for better-informed and highly accurate
106 alignments (Figure 1). CCMetagen is then used to perform quality filtering and produce taxonomic
107 classifications that can be explored in text or interactive visualization formats (Krona plots - Ondov et
108 al. 2011). Our pipeline uses the NCBI taxonomic database (taxids) to produce ranked and updated
109 taxonomic classifications, so that the ever-changing species nomenclature issue is minimized
110 (Federhen 2012). CCMetagen yields classifications at a taxonomic level that reflect the similarity
111 between query and reference sequences. This ranked classification means that the method is able to
112 identify species that have only distant relatives in reference databases (*e.g.* undescribed genera), as
113 well as well-known microorganisms. The output of CCMetagen can be easily converted into a
114 PhyloSeq object for statistical analyses in R (McMurdie and Holmes 2013). The pipeline is sufficiently
115 fast to use the entire NCBI nucleotide collection (nt) as a reference database, thereby enabling the

116 inclusion of microbial eukaryotes – in addition to bacteria, viruses and archaea – in metagenome
117 surveys. Our program is implemented in Python 3 and is freely available at
118 https://github.com/vrmarcelino/CCMetagen.
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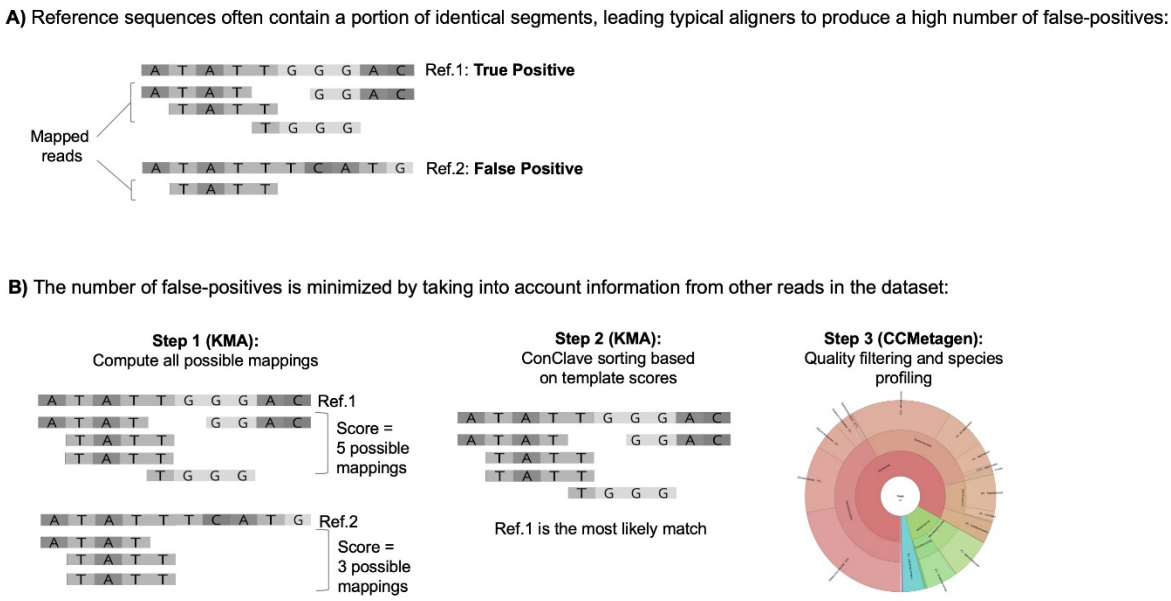

120
121 **Figure 1**. Overview of the ConClave sorting scheme applied to species identification in metagenomic
122 data sets. The figure represents a data set containing 5 sequence reads (4bp) and two closely-related
123 reference sequences (templates), including a true positive (Ref.1) and a potential false positive
124 (Ref.2). (A) Commonly used read mappers yield a high number of false-positives because reads can
125 be randomly assigned to closely-related reference sequences sharing identical fragments spanning
126 the whole sequence read (represented by the ATATT region). (B) The KMA aligner minimizes this
127 problem by scoring reference sequences based on all possible mappings of all reads, and then
128 choosing the templates with the highest scores. Coupled with KMA, CCMetagen produces highly
129 accurate taxonomic assignments of reads in metagenomic data sets in user-friendly formats.
130
131
132 *Fungal classifications are more accurate with the CCMetagen pipeline*
133 To test the performance of CCMetagen in identifying an important and diverse group of
134 microbial eukaryotes, we simulated *in silico* a fungal metatranscriptome (15 species) and a fungal

135 metagenome (30 species). We then benchmarked CCMetagen's performance by comparing it with
136 widely used metagenomic classification software, including Centrifuge (Kim et al. 2016), Kraken2
137 (Wood and Salzberg 2016) and KrakenUniq (Breitwieser et al. 2018). These programs were chosen
138 because they are compatible with custom-made reference databases, which is a desirable flexibility
139 when working with microbial eukaryotes. KrakenUniq was recently shown to outperform eleven other
140 classification methods when using the NCBI nucleotide collection ('nt' database), including
141 Diamond/Blast + MEGAN (Altschul et al. 1990; Huson et al. 2007; Buchfink et al. 2015), CLARK
142 (Ounit et al. 2015), GOTTCHA (Freitas et al. 2015), PhyloSift (Darling et al. 2014) and MetaPhlAn2
143 (Truong et al. 2015). KrakenUniq therefore provides a gold standard for the available tools. We
144 evaluated precision, recall and F1 scores of the benchmarked software in identifying fungal taxa in the
145 simulated fungal metagenome and metatranscriptome (see Methods). The F1 score is the harmonic
146 average of precision and recall; high F1 scores can be interpreted as a good trade-off between
147 precision and recall.
148 The CCMetagen pipeline achieved the highest precision and F1 scores of all the approaches
149 tested (Figure 2, Supplemental Table S1, Supplemental Figures S1 and S2). KrakenUniq achieved
150 higher precision than Kraken2 and Centrifuge when using an ideal database (*i.e.* RefSeq-bf, which
151 contains only the complete and curated genomes of fungi and bacteria, containing all species from
152 the test data set). However, the performance of KrakenUniq decreased substantially when the
153 database was incomplete (*i.e.* RefSeq-f-partial, where a part of the reference sequences was
154 removed to mimic the effects of handling species without reference genomes).
155

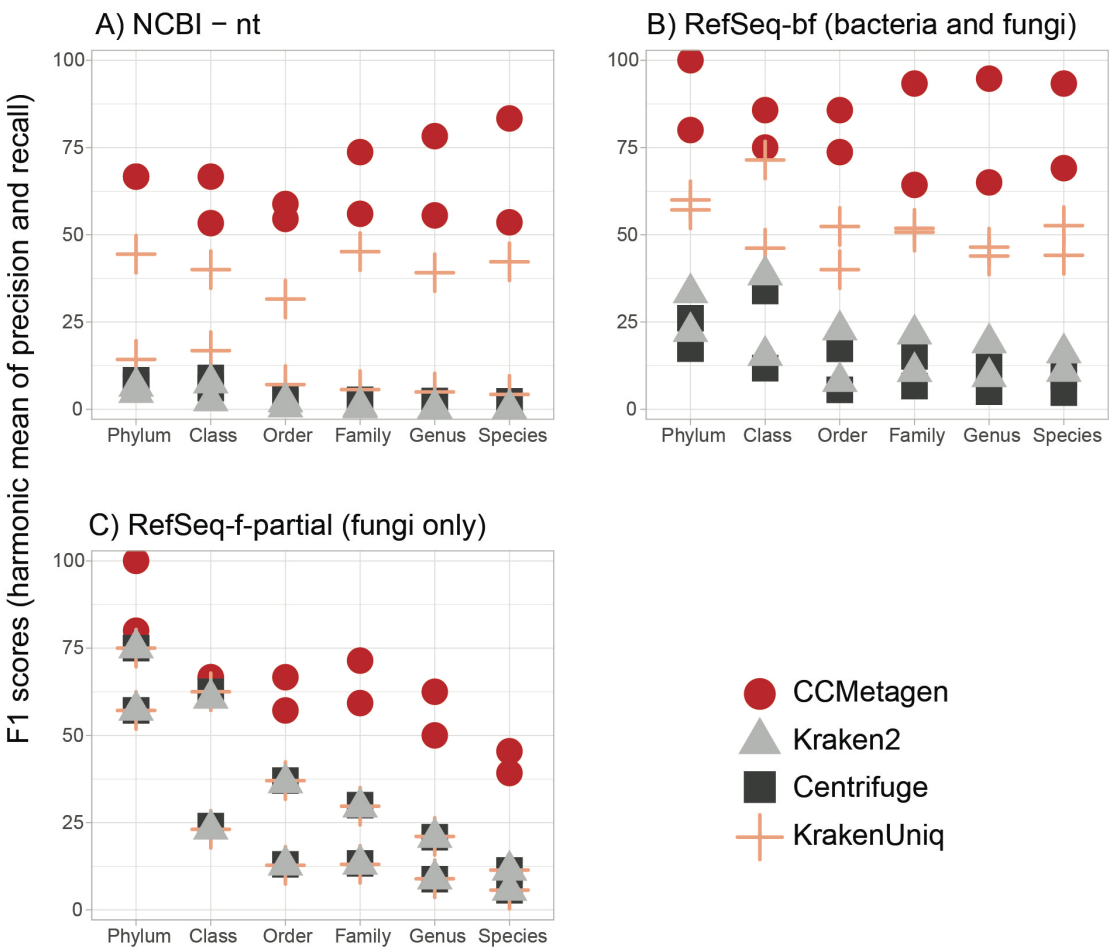

Figure 2. The CCMetagen pipeline has a higher F1 score than other metagenomic classification methods for all taxonomic ranks. The two points for each program and taxonomic rank represent the results using a simulated metagenome and a metatranscriptome sample of a fungal community. (A) Results using the whole NCBI nt collection as a reference database. (B) Results using the RefSeq-bf (bacteria and fungi) database, containing all bacterial and fungal genomes available. (C) Partial RefSeq database containing only some of the fungal species currently present in the RefSeq-bf database, mimicking the effects of dealing with species without representatives in reference data sets. In this case, Kraken2, Centrifuge and KrakenUniq have overlapping results. Refer to Supplemental Table S1 and Supplemental Figures S1 and S2 for more information, including precision and recall.

Centrifuge, Kraken2 and KrakenUniq yielded many more taxa than included in the test data sets: for example, Centrifuge, when used with the nt database, reported 6950 species in the simulated metagenome containing 30 species, while CCMetagen yielded only 15. Naturally, their

172 recall was very high – Centrifuge and KrakenUniq recovered 100% of the taxa present in the test data
173 set when using the RefSeq-bf and nt reference databases (Supplemental figure S2). The species-
174 level recall of Kraken2 decreased when using the nt database. CCMetagen recovered between 50%
175 and 100% of the species when used with RefSeq-bf and nt databases (Supplemental Table S1).
176 The fastest processing time was achieved by Kraken2 (Table 1). The combined CPU time of
177 KMA and CCMetagen (*i.e.* the CCMetagen pipeline) was faster than Centrifuge and KrakenUniq when
178 using the whole NCBI nt database, but it was the slowest approach when using the RefSeq database.
179 The KMA indexing of the nt database was limited to only include *k*-mers with a two-letter prefix, which
180 on average corresponds to only saving non-overlapping *k*-mers. This prefixing substantially increases
181 the speed and could also be applied to the RefSeq database if faster processing time is required
182 (Supplemental Materials). When the NCBI nt data set was used, CCMetagen required ~ 15min to
183 process a sample (~5GB, 7.8M reads on average).
184
185 **Table 1.** CPU time (in minutes) required to analyze a simulated fungal metatranscriptome (mtt) and a
186 fungal metagenome (mtg).

	nt		**RefSeq-bf**		**RefSeq-f-Partial**	
	mtt	mtg	mtt	mtg	mtt	mtg
Kraken2	10.92	7.05	5.29	3.98	4.48	3.50
CCMetagen*	17.24	13.54	85.74	67.00	69.29	20.58
Centrifuge	40.11	27.54	23.70	19.41	16.67	16.10
KrakenUniq	74.11	74.94	43.33	40.85	29.65	21.04

187 * The CCMetagen time was calculated as the sum of the CPU time used by KMA and CCMetagen.
188
189
190 *Bacterial communities are best depicted with the CCMetagen pipeline*
191 We assessed the performance of the CCMetagen pipeline with 10 bacterial communities simulated at
192 different levels of complexity (Segata et al. 2012; McIntyre et al. 2017). Using the NCBI nt collection
193 as reference, CCMetagen achieved the highest precision and F1 scores, at all taxonomic ranks
194 (Figure 3). Recall was highest for Centrifuge and KrakenUniq. In this data set, the recall of Kraken2

195 was higher than CCMetagen from phylum to family-level classifications, but lower than CCMetagen at
196 genus and species level.
197 The complete CCMetagen pipeline (KMA + CCMetagen) required an average of 2.1 minutes
198 to process the bacterial metagenomes (+/- 0.26 SD). It was slower than Kraken2 (average 0.27m, +/-
199 0.21 SD) and faster than KrakenUniq (average 2.56m, +/- 2.60 SD) and Centrifuge (average 9.19m,
200 +/- 0.80 SD).
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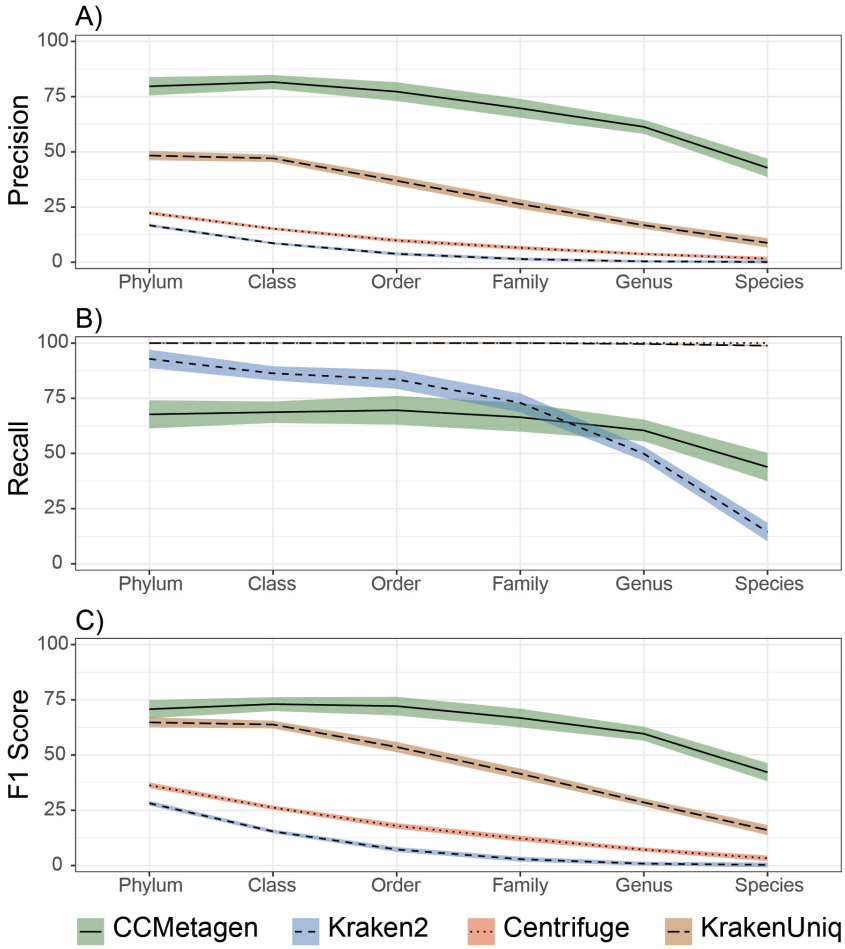

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204 **Figure 3.** CCMetagen pipeline performance for bacterial classifications, compared with Kraken2,
205 Centrifuge and KrakenUniq. Precision (% of true positives), Recall (% of taxa identified) and F1
206 scores represent averages across 10 simulated metagenome samples. Shaded areas indicate 75%
207 confidence intervals.
208
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Biological data set 1: experimentally seeded fungal metatranscriptome

We validated the CCMetagen pipeline with a fungal community previously generated *in vitro* by culturing, processing and sequencing 15 fungal species (Marcelino et al. 2019a, Supplemental Table S2). The analyses were performed using the NCBI nt collection as reference. Our pipeline correctly retrieved 13 out of the 15 fungal species sequenced, in addition to identifying a small component of other eukaryotic (0.4%) and bacterial (3%) RNA, which likely represents laboratory contaminants (Figure 4, Supplemental Table S3).

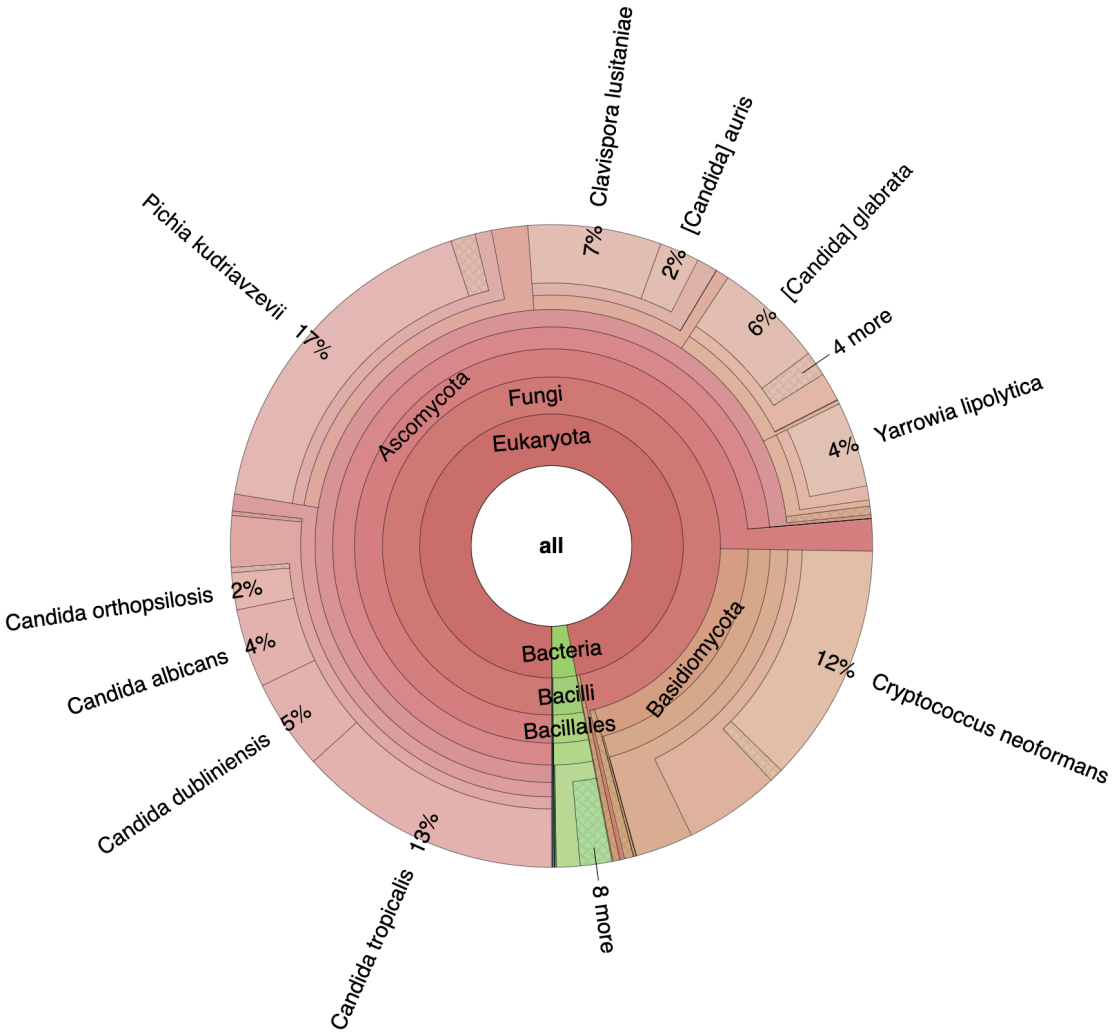

Figure 4. Snapshot of CCMetagen results for a spiked fungal community. This Krona graph shows the relative abundance of taxa at various taxonomic levels, which are color-coded according to their taxonomic classification at lower-ranks – here we see fungal taxa in shades of red, and bacterial taxa in shades of green. The Krona html file can be opened and interactively inspected in a web browser. Each circle represents a taxonomic level, where the user can click for a representation of the relative abundance at a given taxonomic rank. For a detailed list of taxa, refer to Supplemental Table S3.

224

225 As this data set contains the same 15 fungal species as those simulated *in silico*, it is possible
226 to tease apart classification errors from laboratory-related confounders such as contamination.
227 Accordingly, we were able to retrieve all 15 species when using the *in silico* data set, suggesting that
228 the two false-negatives (*Schizosaccharomyces pombe* and *Debaryomyces hansenii*) were missing
229 due to laboratory-related issues, such as RNA extraction biases, gene [under]expression and
230 imprecise cell counts. We also identified seven times more false-positives in the seeded fungal
231 metatranscriptome (44 species, while the simulated data yielded only 6). These additional 38 species
232 were present at low abundance and most likely represent reagent and laboratory contaminants (Salter
233 et al. 2014; Strong et al. 2014).

234

235

236 *Biological data set 2: the microbiome of Australian birds*

237 We used the CCMetagen pipeline to characterize the gut microbiome associated with 9
238 metatranscriptome libraries from wild birds sampled at various sites across Australia (Wille et al.
239 2018; Marcelino et al. 2019b). Fungal and bacterial transcripts were observed in all libraries
240 (Supplemental Table S4). Eukaryotic microbes accounted for 60% of the family-level diversity of the
241 bird microbiome samples (taxa unclassified at family-level were not taken into account). Notably, fungi
242 represented 12 of the 20 most abundant microbial families, surpassing the diversity of bacterial
243 families (Figure 5). Among the fungal transcripts with a species-level classification, those attributed to
244 the basidiomycete *Cystofilobasidium macerans* (Tremellomycetes) were the most abundant and were
245 present in all bird libraries. Transcripts from species of *Mucor*, *Cladosporium*, *Metschnikowia,*
246 *Fusarium* and *Cryptococcus* were common. Other microbial eukaryotes were also observed, including
247 the trichomonad *Simplicimonas* and the Apicomplexan *Eimeria*. Archaeal and viral transcripts were
248 also detected. The methanogenic archaea *Methanobrevibacter woesei*, which was previously
249 reported in chicken guts (Saengkerdsub et al. 2007), was observed in two duck libraries. Influenza A
250 virus was detected and confirmed with PCR-based methods (Wille et al. 2018). The CCMetagen
251 results were parsed with PhyloSeq for a graphical representation of the most abundant microbes, and
252 the R script to reproduce Figure 5 is available on the CCMetagen website.

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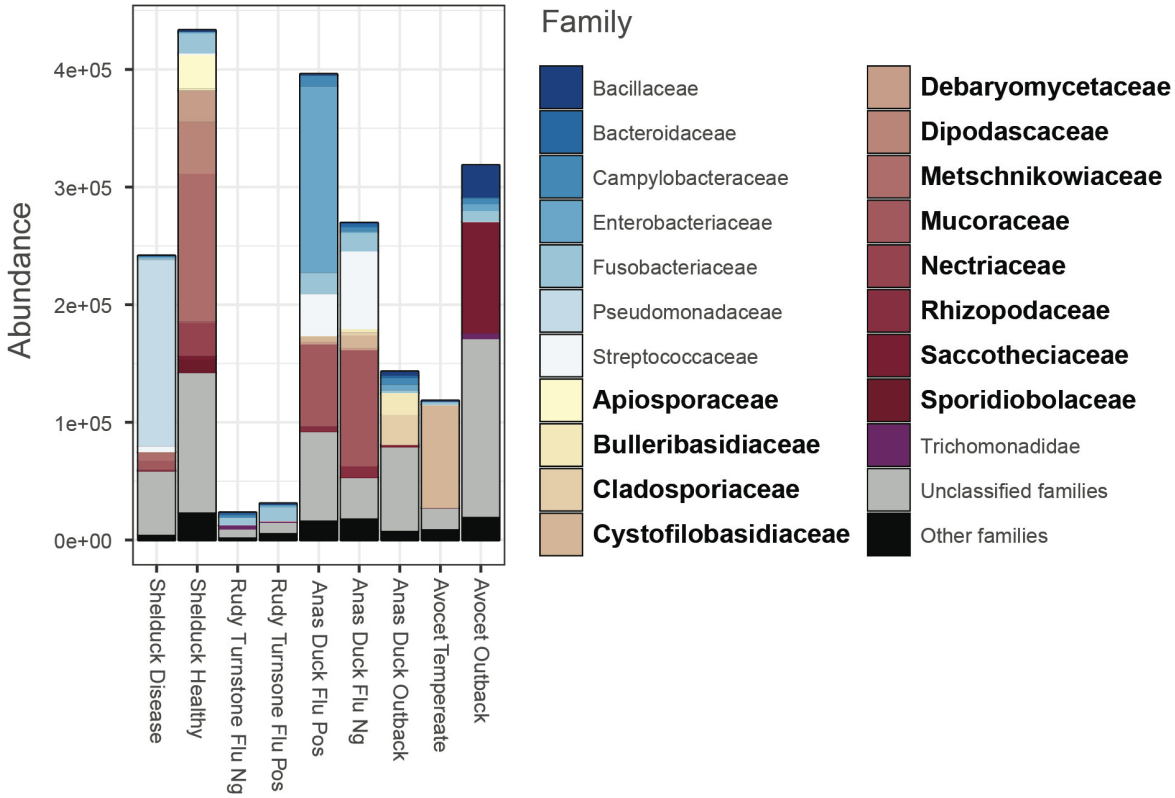

Figure 5. Microbial families in the microbiome of wild birds. The 20 most abundant families are shown, with fungal families indicated in bold. For a full list of taxa, refer to Supplemental Table S4. A tutorial and R scripts to reproduce these analyses are available on the CCMetagen website.

Discussion

The application of the ConClave sorting scheme to differentiate highly similar genetic sequences (Clausen et al. 2018) represents an important step forward in metagenomic species profiling. We have applied this concept to develop a metagenome classification pipeline that is highly accurate yet fast enough to use the entire NCBI nucleotide collection as reference, thereby facilitating the identification of microbial eukaryotes in metagenomic studies. The species-level identifications of bacteria and fungi obtained with the CCMetagen pipeline were from 3× to 1580× more precise than other metagenome classifiers (across all databases tested). CCMetagen is therefore a powerful tool for achieving accurate taxa identifications across a range of biological kingdoms in metagenome or metatranscriptome samples.

271 Scarce reference data pose a major challenge to study any microbial system that is less well-
272 studied than the human gut. Some of the methods with reportedly high accuracy rely heavily on
273 reference databases of complete or near complete genomes. KrakenUniq, for example, showed
274 relatively high precision and recall when using the RefSeq-bf database, which contained the complete
275 genomes of all species in the test data set. However, when KrakenUniq was tested with an
276 incomplete reference database (RefSeq-f-partial), the number of false positives increased, on
277 average, from 51 to 221 species. This likely happens because it is relatively easy to identify a species
278 that is present in the reference database, while it can be challenging to identify the closest match in
279 the absence of a perfectly matching reference sequence. In the latter case, when reads are classified
280 individually, multiple reference sequences can have identical levels of similarity, leading to a high
281 number of false-positives. This is an obvious problem when working with microbial eukaryotes, for
282 which very few complete genomes are available.
283 One of the many advantages of metagenomics is that it enables the detection of novel and
284 rare microbes. Being able to distinguish between known and novel microorganisms in metagenomic
285 data sets is a desirable feature possessed by surprisingly few metagenome classifiers. Some of these
286 classifiers (e.g. MEGAN and Kraken) use the lowest common ancestor between all reference
287 sequences that match the query sequence. The accuracy of these taxonomic classifiers tends to
288 decrease as reference databases get populated with closely-related taxa (Nasko et al. 2018) and,
289 paradoxically, well-known taxa can be classified at higher taxonomic ranks than rare or novel ones.
290 CCMetagen classifies taxa at the lowest common ancestor that reflects the genetic similarity between
291 the query and the reference sequence. As rates of molecular evolution can vary substantially among
292 genes and species, it is currently not feasible to set a universal sequence similarity threshold that
293 works equally well for all organisms and genes. By default, CCMetagen uses similarity thresholds
294 previously determined for fungi (Vu et al. 2016; Vu et al. 2019). Importantly, CCMetagen allows the
295 user to easily set different similarity thresholds or disable the threshold-filtering step entirely. While
296 this strategy also has limitations, it is a better alternative to the reference-dependent method of
297 calculating LCAs, even when using the default thresholds for bacterial classifications (Figure 3).
298 With CCMetagen, it is possible to confidently use metagenomics to identify microbial
299 eukaryotes and prokaryotes in microbial communities. Our analyses of the gut microbiome of wild
300 birds revealed an abundant and diverse community of micro-eukaryotes, representing 60% of the

301 family-level diversity in the samples. We detected various species of *Mucor* and of basidiomycetes,
302 including species of the opportunistic pathogen genus *Cryptococcus*. These and other non-
303 ascomycetes fungi can be affected by mismatches in commonly used metabarcoding primers
304 (Bellemain et al. 2010; Ihrmark et al. 2012; Tedersoo and Lindahl 2016). The fact that they were
305 observed in high abundance indicates that metagenomics and metatranscriptomics are valuable for
306 detecting these organisms in environmental samples. Importantly, CCMetagen can generate results in
307 a format that resembles an Operational Taxonomic Unit (OTU) table that can be imported into
308 software designed for microbial community analyses, such as PhyloSeq (McMurdie and Holmes
309 2013), facilitating downstream ecological and statistical analyses of the microbiome.
310 In summary, CCMetagen is a versatile pipeline implementing the ConClave sorting scheme
311 (via KMA) to achieve highly accurate taxonomic classifications. The pipeline is fast enough to use the
312 entire NCBI nt collection as reference, facilitating the inclusion of understudied organisms, such as
313 microbial eukaryotes, in metagenome surveys. CCMetagen then produces ranked taxonomic results
314 in user-friendly formats that are ready for publication (with Krona) or for downstream statistical
315 analyses (with PhyloSeq). We expect that a range of novel ecological and evolutionary insights will be
316 obtained as information about microbial eukaryotes in metagenomic studies becomes more
317 accessible.
318
319
320 **Methods**
321
322 *Test data sets*
323 A fungal metagenome and a metatranscriptome were simulated *in silico* to assess the
324 performance of CCMetagen and other classification pipelines in identifying the fungal members of a
325 microbial community (Supplemental Table S2). Simulations were based on complete fungal genomes
326 obtained from the NCBI RefSeq collection (Pruitt et al. 2007). The metagenome contained 30 fungal
327 species and was simulated with Grinder (Angly et al. 2012) using parameters to mimic the insert size
328 and sequencing errors of an Illumina library (-md poly4 3e-3 3.3e-8 -insert_dist 500 normal 50 -fq 1 -ql
329 30 10). Coverage was set to vary between 0.001× and 10× for different species. The
330 metatranscriptome contained 15 fungal species and was simulated for a subsample of 4000 genes

331 (CDSs) from each fungal genome. Transcripts were simulated with Polyester (Frazee et al. 2015),
332 using the Illumina5 error model and gene expression following a normal distribution of average 3×
333 (20% of genes up- and 20% down-regulated).
334 Additionally, 10 bacterial metagenomes simulated by Segata et al. (2012), and compiled in
335 McIntyre et al. (2017), were used to assess the performance of the different classifiers in identifying
336 prokaryotic communities with various levels of complexity. Each metagenome contained between 25
337 and 100 bacterial species.
338
339 *Reference databases*
340 We used three reference databases: (i) "nt" - the NCBI nucleotide collection; (ii) "RefSeq-bf",
341 containing curated genomes of fungi (all assembly levels) and bacteria (only complete) in NCBI
342 Reference Sequence Database; and (iii) "RefSeq-f-partial", which is a subset of RefSeq-bf, containing
343 only part of the fungal species in our test data sets. The RefSeq-f-partial database was built to assess
344 how the programs perform when reference databases are incomplete, for example, when dealing with
345 species without reference genomes. Fifteen species were removed, resulting in a database that
346 contained 15 of the 30 species in the fungal metagenome sample, and 7 of the 15 species in the
347 metatranscriptome sample (species removed from this data set are detailed in Supplemental Table
348 S5). Details about databases download and indexing can be found in the Supplemental Material. The
349 nt and RefSeq-bf databases indexed to function with KMA and CCMetagen are hosted in two sites, at
350 https://cloudstor.aarnet.edu.au/plus/s/Mp8gLimDYoLfelH (Australia) and
351 http://www.cbs.dtu.dk/public/CGE/databases/CCMetagen/ (Denmark).
352
353
354 *Benchmarking*
355 Details about the quality control and data analyses are described in the Supplemental
356 Materials. Metagenome classifications using Kraken2 v.2.0.6-beta, KrakenUniq v.0.5.6 and Centrifuge
357 v.1.0.3-beta were performed using default values. The performance of the classifiers was assessed in
358 terms of precision, recall, F1 score and CPU time. Precision was calculated with the formula:
359
360

$$Precision = \frac{True\ Positives}{True\ Positives + False\ Positives}$$

361

362 Recall was calculated with the formula:

363

364 $$Recall = \frac{True\ Positives}{True\ Positives + False\ Negatives}$$

365

366 And the F1 score, which is the harmonic average of the precision and recall, was calculated

367 as:

368

369 $$F1 = 2\ x\ \frac{Precision \times Recall}{Precision + Recall}$$

370

371 Precision and Recall were multiplied by 100 to indicate percentages. CPU time was

372 calculated with GNU's time function (user + sys). True and false positives, at several taxonomic levels

373 (superkingdom to species), were calculated based on NCBI taxids. Only matches to organisms with

374 valid taxids were included in the analyses. Valid but obsolete taxids (those that have changed due to

375 nomenclature changes) were updated accordingly using the ETE toolkit (Huerta-Cepas et al. 2016).

376 This strategy also minimizes nomenclature problems. For example: *Filobasidiella neoformans* is a life

377 stage of *Cryptococcus neoformans*, they share a unique taxid (5207) regardless of the name

378 attributed to the sequence in the reference database. The benchmarking scripts are available at:

379 https://github.com/vrmarcelino/CCMetagen/tree/master/BenchmarkingTools.

380

381 *CCMetagen applied to real data sets*

382 We validated the CCMetagen pipeline using two biological data sets: one defined fungal

383 community (biological data set 1) and one set of environmental samples (biological data set 2). The

384 fungal community was constructed by culturing, pooling and sequencing the same 15 fungal species

385 used in the metatranscriptome simulated *in silico* (SRA BioProject number PRJNA521097) (Marcelino

386 et al. 2019a).

387 The biological data set 2 consisted of nine metatranscriptome libraries derived from gut

388 samples from Australian wild birds (SRA BioProject number PRJNA472212) (Wille et al. 2018).

389 Quality control was performed as described in Marcelino et al. (2019b).

These samples were mapped to the NCBI nucleotide database using KMA with the options -1t1 -mem_mode -and -apm f, and then processed with CCMetagen using default values. The results were parsed with PhyloSeq to produce a graph with relative abundances (Figure 5). A tutorial explaining the full analyses of the bird microbiome, from quality control to graphical representation with PhyloSeq, is available at https://github.com/vrmarcelino/CCMetagen/tree/master/tutorial.

Data access

CCMetagen is freely available from https://github.com/vrmarcelino/CCMetagen (licensed under GNU General Public License v3.0). The simulated fungal metagenome and metatranscriptome sequence is available at https://cloudstor.aarnet.edu.au/plus/s/Mp8gLimDYoLfelH

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