OptiFit: an improved method for fitting amplicon sequences to existing OTUs

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Kelly L. Sovacool[1], Sarah L. Westcott[2], M. Brodie Mumphrey[1], Gabrielle A. Dotson[1], Patrick D. Schloss[2,3,†]

1 Department of Computational Medicine and Bioinformatics, University of Michigan

2 Department of Microbiology and Immunology, University of Michigan

3 Center for Computational Medicine and Bioinformatics, University of Michigan

† To whom correspondence should be addressed: pschloss@umich.edu

1 **Abstract**

Assigning amplicon sequences to operational taxonomic units (OTUs) is often an important step in characterizing the composition of microbial communities across large datasets. OptiClust, a *de novo* OTU clustering method, has been shown to produce higher quality OTU assignments than other methods and at comparable or faster speeds. A notable difference between *de novo* clustering and database-dependent reference clustering methods is that OTU assignments from *de novo* methods may change when new sequences are added to a dataset. However, in some cases one may wish to incorporate new samples into a previously clustered dataset without performing clustering again on all sequences, such as when comparing across datasets or deploying machine learning models where OTUs are features. Existing reference-based clustering methods produce consistent OTUs, but they only consider the similarity of each query sequence to a single reference sequence in an OTU, thus resulting in OTU assignments that are significantly worse than those generated by *de novo* methods. To provide an efficient and robust method to fit amplicon sequence data to existing OTUs, we developed the OptiFit algorithm. Inspired by OptiClust, OptiFit considers the similarity of all pairs of reference and query sequences in an OTU to produce OTUs of the best possible quality. We tested OptiFit using four microbiome datasets with two different strategies: by clustering to an external reference database or by splitting the dataset into a reference and query set and clustering the query sequences to the reference set after clustering it using OptiClust. The result is an improved implementation of closed and open-reference clustering. OptiFit produces OTUs of similar quality as OptiClust and at faster speeds when using the split dataset strategy, although the OTU quality and processing speed depends on the database chosen when using the external database strategy. OptiFit provides a suitable option for users who require consistent OTU assignments at the same quality afforded by *de novo* clustering methods.

Importance

Advancements in DNA sequencing technology have allowed researchers to affordably generate millions of sequence reads from microorganisms in diverse environments. Efficient and robust software tools are needed to assign microbial sequences into taxonomic groups for characterization and comparison of communities. The OptiClust algorithm produces high quality groups by comparing sequences to each other, but the assignments can change when new sequences are added to a dataset, making it difficult to compare different studies. Other approaches assign sequences to groups by comparing them to sequences in a reference database to produce consistent assignments, but the quality of the groups produced is reduced compared to OptiClust. We developed OptiFit, a new reference-based algorithm that produces consistent yet high quality assignments like OptiClust. OptiFit allows researchers to compare microbial communities across different studies or add new data to existing studies without sacrificing the quality of the group assignments.

Introduction

Amplicon sequencing is a mainstay of microbial ecology. Researchers can affordably generate millions of sequences to characterize the composition of hundreds of samples from microbial communities without the need for culturing. In many analysis pipelines, 16S rRNA gene sequences are assigned to operational taxonomic units (OTUs) to facilitate comparison of taxonomic composition between communities to avoid the need for taxonomic classification. A distance threshold of 3% (or sequence similarity of 97%) is commonly used to cluster sequences into OTUs based on pairwise comparisons of the sequences within the dataset. The method chosen for clustering affects the quality of OTU assignments and thus may impact downstream analyses of community composition (1–3).

There are two main categories of OTU clustering algorithms: *de novo* and reference-based. OptiClust is a *de novo* clustering algorithm which uses the distance score between all pairs of sequences in the dataset to cluster them into OTUs by maximizing the Matthews Correlation Coefficient (MCC) (1). This approach takes into account the distances between all pairs of sequences when assigning query sequences to OTUs, in contrast to other *de novo* methods such as the greedy clustering algorithms implemented in USEARCH and VSEARCH (4, 5). In methods employing greedy clustering algorithms, only the distance between each sequence and a representative centroid sequence in the OTU is considered while clustering. As a result, distances between pairs of sequences in the same OTU are frequently larger than the specified threshold, i.e. they are false positives. In contrast, the OptiClust algorithm takes into account the distance between all pairs of sequences when considering how to cluster sequences into OTUs and is thus less willing to take on false positives. A limitation of *de novo* clustering is that different OTU assignments will be produced when new sequences are added to a dataset, making it difficult to use *de novo* clustering to compare OTUs between different studies. Furthermore, since *de novo* clustering requires calculating and comparing distances between all sequences in a

67   dataset, the execution time can be slow and memory requirements can be prohibitive for

68   very large datasets. Reference clustering attempts to overcome the limitations of *de novo*

69   clustering methods by using a representative set of sequences from a database, with each

70   reference sequence seeding an OTU. Commonly, the Greengenes set of representative full

71   length sequences clustered at 97% similarity is used as the reference with VSEARCH (5–7).

72   Query sequences are then clustered into OTUs based on their similarity to the reference

73   sequences. Any query sequences that are not within the distance threshold to any of

74   the reference sequences are either thrown out (closed reference clustering) or clustered

75   *de novo* to create additional OTUs (open reference clustering). While reference-based

76   clustering is generally fast, it is limited by the diversity of the reference database. Novel

77   sequences in the sample will be lost in closed reference mode if they are not represented

78   by a similar sequence in the database. Previous studies found that the OptiClust *de novo*

79   clustering algorithm created the highest quality OTU assignments of all clustering methods

80   (1).

81   To overcome the limitations of current reference-based and *de novo* clustering algorithms

82   while maintaining OTU quality, we developed OptiFit, a reference-based clustering

83   algorithm. While other tools represent reference OTUs with a single sequence, OptiFit

84   uses multiple sequences in existing OTUs as the reference and fits new sequences to

85   those reference OTUs. In contrast to other tools, OptiFit considers all pairwise distance

86   scores between reference and query sequences when assigning sequences to OTUs

87   in order to produce OTUs of the highest possible quality. Here, we tested the OptiFit

88   algorithm with the reference as a public database (e.g. Greengenes) or *de novo* OTUs

89   generated using a reference set from the full dataset and compared the performance to

90   existing tools. To evaluate the OptiFit algorithm and compare to existing methods, we used

91   four published datasets isolated from soil (8), marine (9), mouse gut (10), and human gut

92   (11) samples. OptiFit is available within the mothur software program.
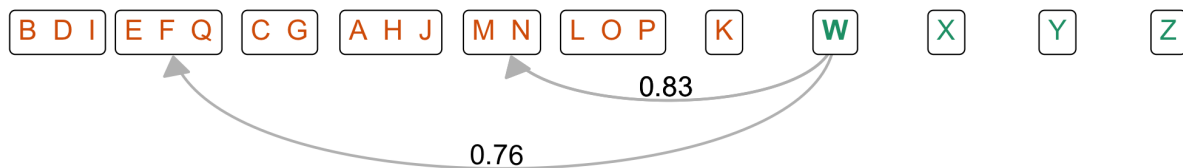
Results

The OptiFit algorithm

OptiFit leverages the method employed by OptiClust of iteratively assigning sequences to OTUs to produce the highest quality OTUs possible, and extends this method for reference-based clustering. OptiClust first seeds each sequence into its own OTU as a singleton. Then for each sequence, OptiClust considers whether the sequence should move to a different OTU or remain in its current OTU, choosing the option that results in a better Matthews correlation coefficient (MCC) (1). The MCC uses all values from a confusion matrix and ranges from negative one to one, with a score of one occurring when all sequence pairs are true positives and true negatives and a score of negative one occurring when all pairs are false positives and false negatives. Sequence pairs that are similar to each other (i.e. within the distance threshold) are counted as true positives if they are clustered into the same OTU, and false negatives if they are not in the the same OTU. Sequence pairs that are not similar to each other are true negatives if they are not clustered into the same OTU, and false positives if they are not in the same OTU. OptiClust iterations continue until the MCC stabilizes or until a maximum number of iterations is reached. This process produces *de novo* OTU assignments with the most optimal MCC given the input sequences.

OptiFit begins where OptiClust ends, starting with a list of reference OTUs and their sequences, a list of query sequences to cluster to the reference OTUs, and the sequence pairs that are within the distance threshold (e.g. 0.03) (Figure 1). Initially, all query sequences are placed into separate OTUs. Then, the algorithm iteratively reassigns the query sequences to the reference OTUs to optimize the MCC. Alternatively, a sequence will remain unassigned if the MCC value is maximized when the sequence is a singleton rather than clustered into a reference OTU. All query and reference sequence pairs are considered when calculating the MCC. This process is repeated until the MCC changes by
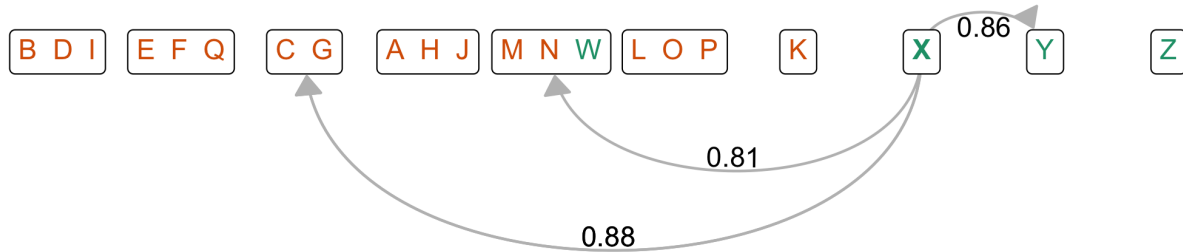
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0. List of sequence pairs within the distance threshold

D	F	G	H	I	I	J	J	N	O	P	P	P	Q	Q	W	W	W	X	X	X	X	Y
B	E	C	A	B	D	A	H	M	L	K	L	O	E	F	F	M	N	C	G	N	Y	C

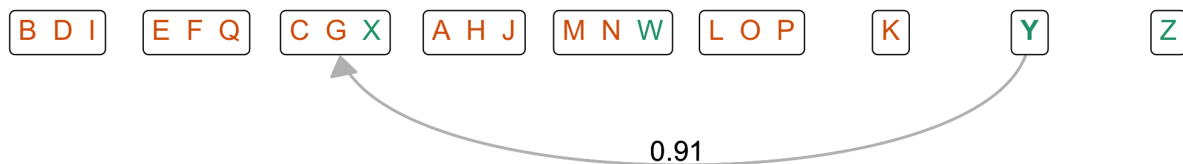*% distance* 1.7 1.4 2.9 2.7 1.7 1.4 1.0 1.6 1.6 2.6 1.5 2.2 2.4 1.8 1.2 2.8 1.0 1.4 2.1 2.7 1.0 2.1 1.4

1. MCC = 0.78

B D I | E F Q | C G | A H J | M N | L O P | K | **W** | X | Y | Z

0.83

0.76

2. MCC = 0.83

B D I | E F Q | C G | A H J | M N W | L O P | K | **X** | Y | Z

0.86

0.81

0.88

3. MCC = 0.88

B D I | E F Q | C G X | A H J | M N W | L O P | K | **Y** | Z

0.91

4. MCC = 0.91

B D I | E F Q | C G X Y | A H J | M N W | L O P | K | **Z**

Figure 1: The OptiFit Algorithm. Here we present a toy example of the OptiFit algorithm fitting query sequences to existing OTUs, given the list of all sequence pairs that are within the distance threshold (here 3% is used). The goal of OptiFit is to assign the query sequences W through Z (colored green) to the reference OTUs created by clustering Sequences A through Q (colored orange) which were previously clustered *de novo* with OptiClust (see the OptiClust supplemental text (1)). Initially, OptiFit places each query sequence in its own OTU. Then, for each query sequence (**bolded**), OptiFit determines what the new MCC score would be if that sequence were moved to one of the OTUs containing at least one other similar sequence. The sequence is then moved to the OTU which would result in the best MCC score. OptiFit stops iterating over sequences once the MCC score stabilizes (in this example; only one iteration over each sequence is needed).

119 no more than 0.0001 (default) or until a maximum number of iterations is reached (default:

120 100). In the closed reference mode, any query sequences that cannot be clustered into

121 reference OTUs are discarded, and the results only contain OTUs that exist in the original

122 reference. In the open reference mode, unassigned query sequences are clustered *de*

123 *novo* using OptiClust to generate new OTUs. The final MCC is reported with the best

124 OTU assignments. There are two strategies for generating OTUs with OptiFit: 1) cluster

125 the query sequences to reference OTUs generated by *de novo* clustering an independent

126 database, or 2) split the dataset into a reference and query fraction, cluster the reference

127 sequences *de novo*, then cluster the query sequences to the reference OTUs.

Reference clustering with public databases

129 To test how OptiFit performs for reference-based clustering, we clustered each dataset to

130 three databases of reference OTUs: the Greengenes database, the SILVA non-redundant

131 database, and the Ribosomal Database Project (RDP) (6, 12, 13). Reference OTUs for

132 each database were created by performing *de novo* clustering with OptiClust at a distance

133 threshold of 3% using the V4 region of each sequence (see Figure 2). After trimming to

134 the V4 region, the databases contained 174,979, 16,192, and 173,648 unique sequences

135 and produced *de novo* MCC scores of 0.72, 0.74, and 0.73 for Greengenes, RDP, and

136 SILVA, respectively. Clustering sequences to Greengenes and SILVA in closed reference

137 mode performed similarly, with median MCC scores of 0.85 and 0.77 respectively, while

138 the median MCC was 0.35 when clustering to RDP (Figure 3). For comparison, clustering

139 datasets with OptiClust produced an average MCC score of 0.87. This gap in OTU quality

140 mostly disappeared when clustering in open reference mode, which produced median

141 MCCs of 0.86 with Greengenes, 0.85 with SILVA, and 0.86 with the RDP. Thus, open

142 reference OptiFit produced OTUs of very similar quality as *de novo* clustering, and closed

143 reference OptiFit followed closely behind as long as a suitable reference database was

144 chosen.
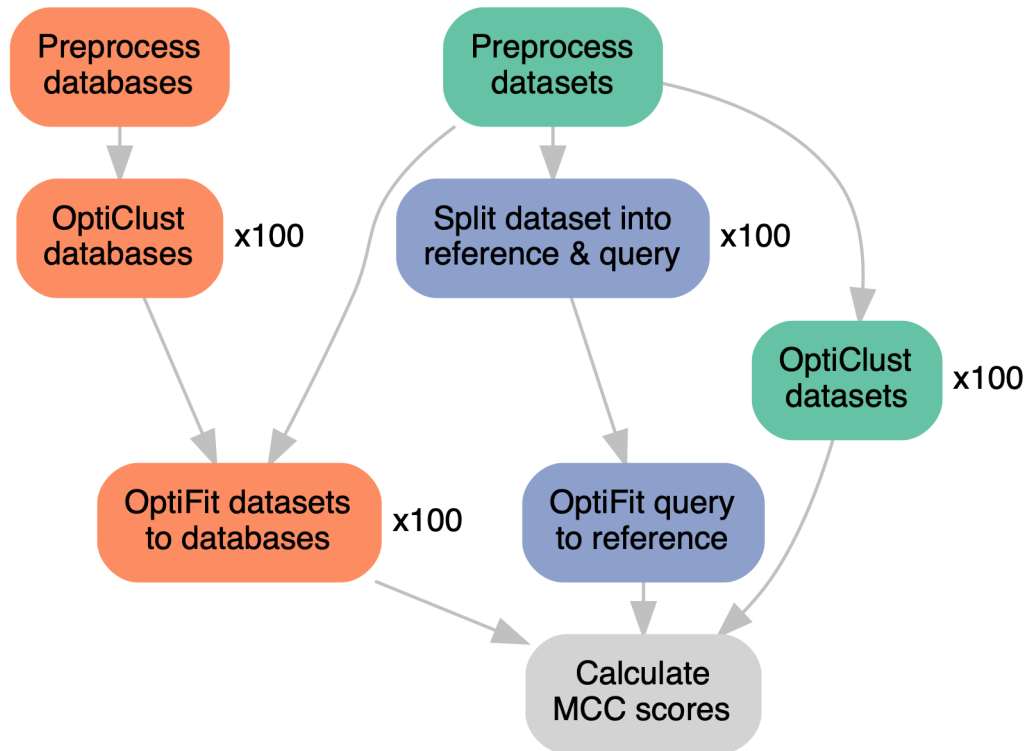
Figure 2: The Analysis Workflow. Reference sequences from Greengenes, the RDP, and SILVA were downloaded, preprocessed with mothur by trimming to the V4 region, and clustered *de novo* with OptiClust for 100 repetitions. Datasets from human, marine, mouse, and soil microbiomes were downloaded, preprocessed with mothur by aligning to the SILVA V4 reference alignment, then clustered *de novo* with OptiClust for 100 repetitions. Individual datasets were fit to reference databases with OptiFit; OptiFit was repeated 100 times for each dataset and database combination. Datasets were also randomly split into a reference and query fraction, and the query sequences were fit to the reference sequences with OptiFit for 100 repetitions. The final MCC score was reported for all OptiClust and OptiFit repetitions.

145 Since closed reference clustering does not cluster query sequences that could not be

146 clustered into reference OTUs, an additional measure of clustering performance to consider

147 is the fraction of query sequences that were able to be clustered. On average, more

148 sequences were clustered with Greengenes as the reference (59.1%) than with SILVA

149 (50.0%) or with the RDP (9.8%) (Figure 3). This mirrored the result reported above that

150 Greengenes produced better OTUs in terms of MCC score than either SILVA or RDP. Note

151 that *de novo* and open reference clustering methods always cluster 100% of sequences

152 into OTUs. The database chosen affects the final closed reference OTU assignments

153 considerably in terms of both MCC score and fraction of query sequences that could be

154 clustered into the reference OTUs.

155 Despite the drawbacks, closed reference methods have been used when fast execution

156 speed is required, such as when using very large datasets (14). To compare performance

157 in terms of speed, we repeated each OptiFit and OptiClust run 100 times and measured

158 the execution time. Across all dataset and database combinations, closed reference OptiFit

159 outperformed both OptiClust and open reference OptiFit (Figure 3). For example, with

160 the human dataset fit to SILVA reference OTUs, the average run times in seconds were

161 406.8 for closed reference OptiFit, 455.3 for *de novo* clustering the dataset, and 559.4 for

162 open reference OptiFit. Thus, the OptiFit algorithm continues the precedent that closed

163 reference clustering sacrifices OTU quality for execution speed.

164 To compare to the reference clustering methods used by QIIME2, we clustered each

165 dataset with VSEARCH against the Greengenes database of OTUs previously clustered

166 at 97% sequence similarity. Each reference OTU from the Greengenes 97% database

167 contains one reference sequence, and VSEARCH maps sequences to the reference

168 based on each individual query sequence's similarity to the single reference sequence.

169 In contrast, OptiFit accepts reference OTUs which each may contain multiple sequences,

170 and the sequence similarity between all query and reference sequences is considered

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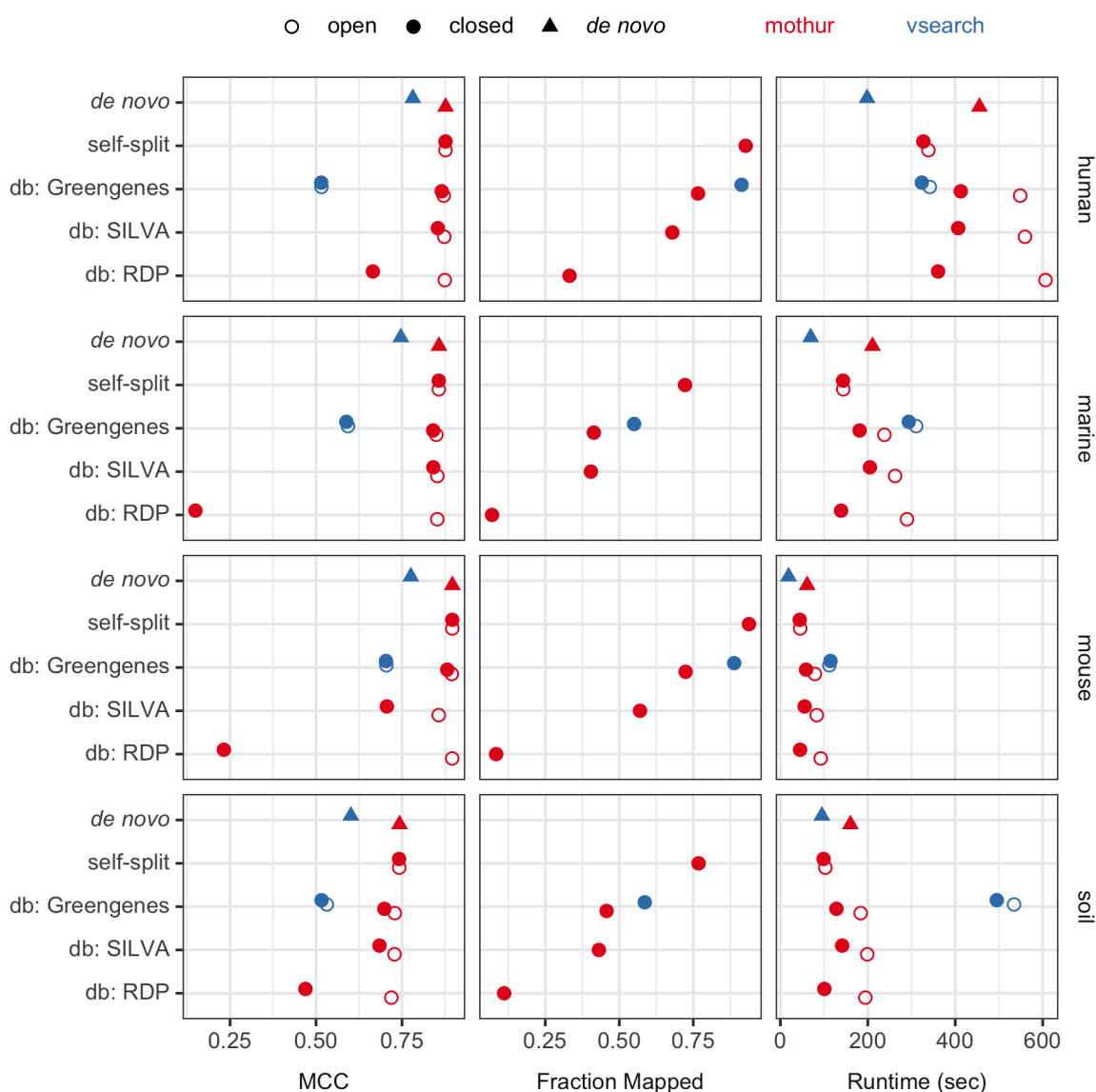
Figure 3: Benchmarking Results. The median MCC score, fraction of query sequences that mapped in closed-reference clustering, and runtime in seconds from repeating each clustering method 100 times. Each dataset underwent *de novo* clustering using OptiClust or reference-based clustering using OptiFit with one of two strategies; splitting the dataset and fitting 50% the sequences to the other 50%, or fitting the dataset to a reference database (Greengenes, SILVA, or RDP). Reference-based clustering was repeated with open and closed mode. For additional comparison, VSEARCH was used for *de novo* and reference-based clustering against the Greengenes database.

171 when assigning sequences to OTUs. In closed reference mode, OptiFit produced 27.2%

172 higher quality OTUs than VSEARCH, but VSEARCH was able to cluster 24.8% more query

173 sequences than OptiFit to the Greengenes reference database (Figure 3). This is because

174 VSEARCH only considers the distances between each query sequence to the single

175 reference sequence, while OptiFit considers the distances between all pairs of reference

176 and query sequences in an OTU. When open reference clustering, OptiFit produced higher

177 quality OTUs than VSEARCH against the Greengenes database, with median MCC scores

178 of 0.86 and 0.56, respectively. In terms of run time, OptiFit outperformed VSEARCH in

179 both closed and open reference mode by 54.6% and 49.5% on average, respectively. Thus,

180 the more stringent OTU definition employed by OptiFit, which prefers the query sequence

181 to be similar to all other sequences in the OTU rather than to only one sequence, resulted

182 in fewer sequences being clustered to reference OTUs than when using VSEARCH, but

183 caused OptiFit to outperform VSEARCH in terms of both OTU quality and execution time.

184 **Reference clustering with split datasets**

185 When performing reference clustering against public databases, the database chosen

186 greatly affects the quality of OTUs produced. OTU quality may be poor when the reference

187 database consists of sequences that are too unrelated to the samples of interest, such as

188 when samples contain novel populations. While *de novo* clustering overcomes the quality

189 limitations of reference clustering to databases, OTU assignments are not consistent when

190 new sequences are added. Researchers may wish to cluster new sequences to existing

191 OTUs or to compare OTUs across studies. To determine how well OptiFit performs for

192 clustering new sequences to existing OTUs, we employed a split dataset strategy, where

193 each dataset was randomly split into a reference fraction and a query fraction. Reference

194 sequences were clustered *de novo* with OptiClust, then query sequences were clustered

195 to the *de novo* OTUs with OptiFit.

First, we tested whether OptiFit performed as well as *de novo* clustering when using the split dataset strategy with half of the sequences selected for the reference by a simple random sample (a 50% split) (Figure 3; self-split). OTU quality was similar to that from OptiClust regardless of mode (0.029% difference in median MCC). In closed reference mode, OptiFit was able to cluster 84.8% of query sequences to reference OTUs with the split strategy, a great improvement over the average 59.1% of sequences clustered to the Greengenes database. In terms of run time, closed and open reference OptiFit performed faster than OptiClust on whole datasets by 34.7% and 33.5%, respectively. The split dataset strategy also performed 13.5% faster than the database strategy in closed reference mode and 43.5% faster in open reference mode. Thus, reference clustering with the split dataset strategy creates as high quality OTUs as *de novo* clustering yet at a faster run time, and fits far more query sequences than the database strategy.

While we initially tested this strategy using a 50% split of the data into reference and query fractions, we next investigated whether there was an optimal reference fraction size. To identify the best reference size, reference sets with 10% to 90% of the sequences were created, with the remaining sequences used for the query (Figure 4). OTU quality was remarkably consistent across reference fraction sizes. For example, splitting the human dataset 100 times yielded a coefficient of variation (i.e. the standard deviation divided by the mean) of 0.00022 for the MCC score across all fractions. Run time generally decreased as the reference fraction increased; for the human dataset, the median run time was 364.1 seconds with 10% of sequences in the reference and 291.3 seconds with 90% of sequences in the reference. In closed reference mode, the fraction of sequences that mapped increased as the reference size increased; for the human dataset, the median fraction mapped was 0.85 with 10% of sequences in the reference and 0.95 with 90% of sequences in the reference. These trends held for the other datasets as well. Thus, the reference fraction did not affect OTU quality in terms of MCC score, but did affect the run time and the fraction of sequences that mapped during the closed reference clustering.

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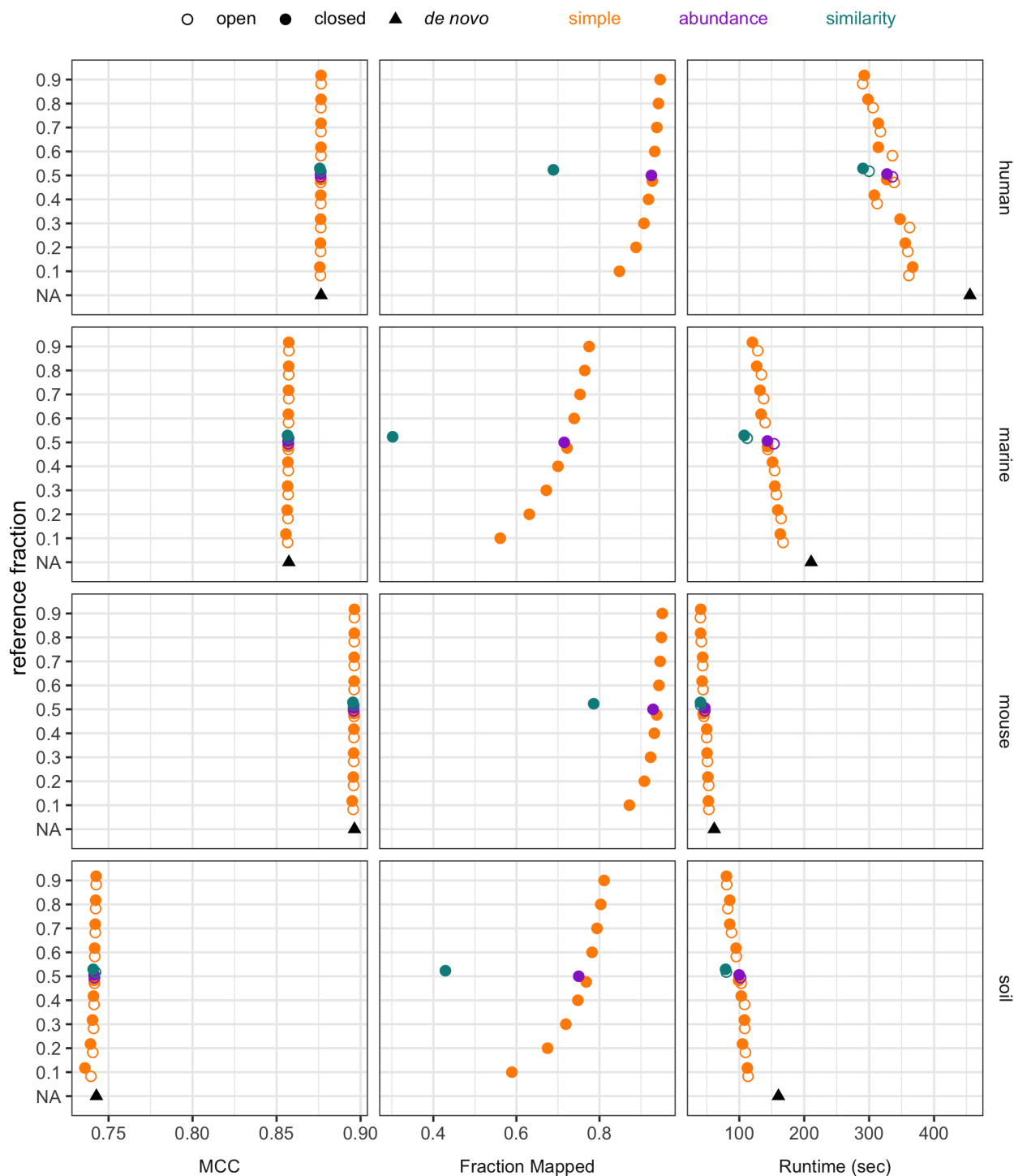
Figure 4: Split dataset strategy. The median MCC score, fraction of query sequences that mapped in closed-reference clustering, and runtime in seconds from repeating each clustering method 100 times. Each dataset was split into a reference and query fraction. Reference sequences were selected via a simple random sample, weighting sequences by relative abundance, or weighting by similarity to other sequences in the dataset. With the simple random sample method, dataset splitting was repeated with reference fractions ranging from 10% to 90% of the dataset and for 100 random seeds. *De novo* clustering each dataset is also shown for comparison.

₂₂₃ After testing the split strategy using a simple random sample to select the reference

₂₂₄ sequences, we then investigated other methods of splitting the data. We tested three

₂₂₅ methods for selecting the fraction of sequences to be used as the reference at a size of

₂₂₆ 50%: a simple random sample, weighting sequences by relative abundance, and weighting

₂₂₇ by similarity to other sequences in the dataset (Figure 4). OTU quality in terms of MCC

₂₂₈ was similar across all three sampling methods (median MCC of 0.87). In closed-reference

₂₂₉ clustering mode, the fraction of sequences that mapped were similar for simple and

₂₃₀ abundance-weighted sampling (median fraction mapped of 0.85 and 0.84, respectively),

₂₃₁ but worse for similarity-weighted sampling (median fraction mapped of 0.56). While simple

₂₃₂ and abundance-weighted sampling produced better quality OTUs than similarity-weighted

₂₃₃ sampling, OptiFit performed faster on similarity-weighted samples with a median runtime of

₂₃₄ 93.8 seconds compared to 123.2 and 122.6 seconds for simple and abundance-weighted

₂₃₅ sampling, respectively. Thus, employing more complicated sampling strategies such as

₂₃₆ abundance-weighted and similarity-weighted sampling did not confer any advantages over

₂₃₇ selecting the reference via a simple random sample, and in fact decreased OTU quality in

₂₃₈ the case of similarity-weighted sampling.

₂₃₉ Discussion

₂₄₀ We developed a new algorithm for clustering sequences to existing OTUs and have

₂₄₁ demonstrated its suitability for reference-based clustering. OptiFit makes the iterative

₂₄₂ method employed by OptiClust available for tasks where reference-based clustering is

₂₄₃ required. We have shown that OTU quality is similar between OptiClust and OptiFit in open

₂₄₄ reference mode, regardless of strategy employed. Open reference OptiFit performs slower

₂₄₅ than OptiClust due to the additional *de novo* clustering step, so users may prefer OptiClust

₂₄₆ for tasks that do not require reference OTUs.

₂₄₇ When clustering to public databases, OTU quality dropped in closed reference mode to

248 different degrees depending on the database and dataset source, and no more than half

249 of query sequences were able to be clustered into OTUs across any dataset/database

250 combination. This may reflect limitations of reference databases, which are unlikely

251 to contain sequences from novel microbes. This drop in quality was most notable

252 with the RDP reference, which contained only 16,192 sequences compared to 173,648

253 sequences in SILVA and 174,979 in Greengenes. Note that Greengenes has not been

254 updated since 2013 at the time of this writing, while SILVA and the RDP are updated

255 regularly. We recommend that users who require an independent reference database

256 opt for large databases with regular updates and good coverage of microbial diversity for

257 their environment. Since OptiClust still performs faster than open reference OptiFit and

258 creates higher quality OTUs than closed reference OptiFit with the database strategy, we

259 recommend using OptiClust rather than clustering to a database whenever consistent

260 OTUs are not required.

261 The OptiClust and OptiFit algorithms produced higher quality OTUs than VSEARCH in

262 open reference, closed reference, or *de novo* modes. However, VSEARCH was able

263 to cluster more sequences to OTUs than OptiFit in closed reference mode. While both

264 OptiFit and VSEARCH use a distance or similarity threshold for determining how to cluster

265 sequences into OTUs, VSEARCH is more permissive than OptiFit regardless of mode.

266 The OptiFit and OptiClust algorithms use all of the sequences to define an OTU, preferring

267 that all pairs of sequences (including reference and query sequences) in an OTU are within

268 the distance threshold in order to maximize the MCC. In contrast, VSEARCH only requires

269 each query sequence to be similar to the single centroid sequence that seeded the OTU.

270 Because of this, VSEARCH sacrifices OTU quality by allowing more dissimilar sequences

271 to be clustered into OTUs.

272 When clustering with the split dataset strategy, OTU quality was remarkably similar when

273 reference sequences were selected by a simple random sample or weighted by abundance,

16

274 but quality was slightly worse when sequences were weighted by similarity. We recommend

275 using a simple random sample since the more sophisticated reference selection methods

276 do not offer any benefit. The similarity in OTU quality between OptiClust and OptiFit with

277 this strategy demonstrates the suitability of using OptiFit to cluster sequences to existing

278 OTUs, such as when comparing OTUs across studies. However, when consistent OTUs

279 are not required, we recommend using OptiClust for *de novo* clustering over the split

280 strategy with OptiFit since OptiClust is simpler to execute but performs similarly in terms of

281 both run time and OTU quality.

282 Unlike existing reference-based methods that cluster query sequences to a single centroid

283 sequence in each reference OTU, OptiFit considers all sequences in each reference OTU

284 when clustering query sequences, resulting in OTUs of a similar high quality as those

285 produced by the *de novo* OptiClust algorithm. Potential applications include clustering

286 sequences to reference databases, comparing taxonomic composition of microbiomes

287 across different studies, or using OTU-based machine learning models to make predictions

288 on new data. OptiFit fills the missing option for clustering query sequences to existing

289 OTUs that does not sacrifice OTU quality for consistency of OTU assignments.

Materials and Methods

Data Processing Steps

292 We downloaded 16S rRNA gene amplicon sequences from four published datasets isolated

293 from soil (8), marine (9), mouse gut (10), and human gut (11) samples. These datasets

294 contain sequences from the V4 region of the 16S rRNA gene and represent a selection

295 of the broad types of natural communities that microbial ecologists study. We processed

296 the raw sequences using mothur according to the Schloss Lab MiSeq SOP (15) and

297 accompanying study by Kozich *et al.* (16). These steps included trimming and filtering

298 for quality, aligning to the SILVA reference alignment (12), discarding sequences that

299 aligned outside the V4 region, removing chimeric reads with UCHIME (17), and calculating

300 distances between all pairs of sequences within each dataset prior to clustering.

Reference database clustering

302 To generate reference OTUs from public databases, we downloaded sequences from the

303 Greengenes database (v13_8_99) (6), SILVA non-redundant database (v132) (12), and the

304 Ribosomal Database Project (v16) (13). These sequences were processed using the same

305 steps outlined above followed by clustering sequences into *de novo* OTUs with OptiClust.

306 Processed reads from each of the four datasets were clustered with OptiFit to the reference

307 OTUs generated from each of the three databases. When reference clustering with

308 VSEARCH, processed datasets were clustered directly to the unprocessed Greengenes

309 97% OTU reference alignment, since this method is how VSEARCH is typically used by

310 the QIIME2 software for reference-based clustering (7, 18).

Split dataset clustering

312 For each dataset, half of the sequences were selected to be clustered *de novo* into

313 reference OTUs with OptiClust. We used three methods for selecting the subset of

314 sequences to be used as the reference: a simple random sample, weighting sequences by

315 relative abundance, and weighting by similarity to other sequences in the dataset. Dataset

316 splitting was repeated with 100 random seeds. With the simple random sampling method,

317 dataset splitting was also repeated with reference fractions ranging from 10% to 90% of

318 the dataset. For each dataset split, the remaining query sequences were clustered into the

319 reference OTUs with OptiFit.

Benchmarking

321 OptiClust and OptiFit randomize the order of query sequences prior to clustering and

322 employ a random number generator to break ties when OTU assignments are of equal

323 quality. As a result, they produce slightly different OTU assignments when repeated

324 with different random seeds. To capture any variation in OTU quality or execution time,

325 clustering was repeated with 100 random seeds for each combination of parameters and

326 input datasets. We used the benchmark feature provided by Snakemake to measure the

327 run time of every clustering job. We calculated the MCC on each set of OTUs to quantify

328 the quality of clustering, as described by Westcott *et al.* (1).

329 **Data and Code Availability**

330 We implemented the analysis workflow in Snakemake (19) and wrote scripts in R (20),

331 Python (21), and GNU bash (22). Software used includes mothur v1.47.0 (23), VSEARCH

332 v2.15.2 (5), the tidyverse metapackage (24), R Markdown (25), ggraph (26), ggtext (27),

333 numpy (28), the SRA toolkit (29), and conda (30). The complete workflow and supporting

334 files required to reproduce this manuscript are available at https://github.com/SchlossLab/

335 Sovacool_OptiFit_2021.

Acknowledgements

Author Contributions

343 KLS wrote the analysis code, evaluated the algorithm, and wrote the original draft of

344 the manuscript. SLW designed and implemented the OptiFit algorithm and assisted in

345 debugging the analysis code. MBM and GAD contributed analysis code. PDS conceived

346 the study, supervised the project, and assisted in debugging the analysis code. All authors

347 reviewed and edited the manuscript.

348 ## References

349 1.   **Westcott SL**, **Schloss PD**. 2017. OptiClust, an Improved Method for Assigning
       Amplicon-Based Sequence Data to Operational Taxonomic Units.   mSphere
350   **2**:e00073–17. doi:10.1128/mSphereDirect.00073-17.

351 2.   **Schloss PD**. 2016. Application of a Database-Independent Approach To Assess the
       Quality of Operational Taxonomic Unit Picking Methods. mSystems **1**:e00027–16.
352   doi:10.1128/mSystems.00027-16.

353 3.   **Westcott SL**, **Schloss PD**. 2015.   De novo clustering methods outperform
       reference-based methods for assigning 16S rRNA gene sequences to operational
354   taxonomic units. PeerJ **3**:e1487. doi:10.7717/peerj.1487.

355 4.   **Edgar RC**. 2010. Search and clustering orders of magnitude faster than BLAST.
356   Bioinformatics **26**:2460–2461. doi:10.1093/bioinformatics/btq461.

357 5.   **Rognes T**, **Flouri T**, **Nichols B**, **Quince C**, **Mahé F**. 2016. VSEARCH: A versatile
358   open source tool for metagenomics. PeerJ **4**:e2584. doi:10.7717/peerj.2584.

359 6.   **DeSantis TZ**, **Hugenholtz P**, **Larsen N**, **Rojas M**, **Brodie EL**, **Keller K**, **Huber
       T**, **Dalevi D**, **Hu P**, **Andersen GL**. 2006. Greengenes, a Chimera-Checked 16S
       rRNA Gene Database and Workbench Compatible with ARB. AEM **72**:5069–5072.
360   doi:10.1128/AEM.03006-05.

361 7.   Clustering sequences into OTUs using Q2-vsearch — QIIME 2 2021.2.0
362   documentation. https://docs.qiime2.org/2021.2/tutorials/otu-clustering/.

363   8.   **Johnston ER**, **Rodriguez-R LM**, **Luo C**, **Yuan MM**, **Wu L**, **He Z**, **Schuur EAG**,
            Luo Y, **Tiedje JM**, **Zhou J**, **Konstantinidis KT**. 2016. Metagenomics Reveals
            Pervasive Bacterial Populations and Reduced Community Diversity across the
364   Alaska Tundra Ecosystem. Front Microbiol **7**. doi:10.3389/fmicb.2016.00579.

365   9.   **Henson MW**, **Pitre DM**, **Weckhorst JL**, **Lanclos VC**, **Webber AT**, **Thrash JC**.
            2016. Artificial Seawater Media Facilitate Cultivating Members of the Microbial
366   Majority from the Gulf of Mexico. mSphere **1**. doi:10.1128/mSphere.00028-16.

367   10.   **Schloss PD**, **Schubert AM**, **Zackular JP**, **Iverson KD**, **Young VB**, **Petrosino JF**.
            2012. Stabilization of the murine gut microbiome following weaning. Gut Microbes
368   **3**:383–393. doi:10.4161/gmic.21008.

369   11.   **Baxter NT**, **Ruffin MT**, **Rogers MAM**, **Schloss PD**. 2016. Microbiota-based model
            improves the sensitivity of fecal immunochemical test for detecting colonic lesions.
370   Genome Med **8**:37. doi:10.1186/s13073-016-0290-3.

371   12.   **Quast C**, **Pruesse E**, **Yilmaz P**, **Gerken J**, **Schweer T**, **Yarza P**, **Peplies J**,
            Glöckner FO. 2013. The SILVA ribosomal RNA gene database project: Improved
            data processing and web-based tools. Nucleic Acids Research **41**:D590–D596.
372   doi:10.1093/nar/gks1219.

373   13.   **Cole JR**, **Wang Q**, **Fish JA**, **Chai B**, **McGarrell DM**, **Sun Y**, **Brown CT**,
            Porras-Alfaro A, **Kuske CR**, **Tiedje JM**. 2014. Ribosomal Database Project: Data
            and tools for high throughput rRNA analysis. Nucl Acids Res **42**:D633–D642.
374   doi:10.1093/nar/gkt1244.

375   14.   **Navas-Molina JA**, **Peralta-Sánchez JM**, **González A**, **McMurdie PJ**, **Vázquez-Baeza Y**, **Xu Z**, **Ursell LK**, **Lauber C**, **Zhou H**, **Song SJ**, **Huntley J**, **Ackermann GL**, **Berg-Lyons D**, **Holmes S**, **Caporaso JG**, **Knight R**. 2013. Chapter Nineteen - Advancing Our Understanding of the Human Microbiome Using

376   QIIME, p. 371–444. *In* DeLong, EF (ed.), Methods in Enzymology. Academic Press.

377   15.   **Schloss PD**, **Westcott SL**. MiSeq SOP. https://mothur.org/MiSeq_SOP.

378

379   16.   **Kozich JJ**, **Westcott SL**, **Baxter NT**, **Highlander SK**, **Schloss PD**. 2013. Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform.

380   Appl Environ Microbiol **79**:5112–5120. doi:10.1128/AEM.01043-13.

381   17.   **Edgar RC**, **Haas BJ**, **Clemente JC**, **Quince C**, **Knight R**. 2011.   UCHIME improves sensitivity and speed of chimera detection. Bioinformatics **27**:2194–2200.

382   doi:10.1093/bioinformatics/btr381.

383   18.   **Bolyen E**, **Rideout JR**, **Dillon MR**, **Bokulich NA**, **Abnet CC**, **Al-Ghalith GA**, **Alexander H**, **Alm EJ**, **Arumugam M**, **Asnicar F**, **Bai Y**, **Bisanz JE**, **Bittinger K**, **Brejnrod A**, **Brislawn CJ**, **Brown CT**, **Callahan BJ**, **Caraballo-Rodríguez AM**, **Chase J**, **Cope EK**, **Da Silva R**, **Diener C**, **Dorrestein PC**, **Douglas GM**, **Durall DM**, **Duvallet C**, **Edwardson CF**, **Ernst M**, **Estaki M**, **Fouquier J**, **Gauglitz JM**, **Gibbons SM**, **Gibson DL**, **Gonzalez A**, **Gorlick K**, **Guo J**, **Hillmann B**, **Holmes S**, **Holste H**, **Huttenhower C**, **Huttley GA**, **Janssen S**, **Jarmusch AK**, **Jiang L**, **Kaehler BD**, **Kang KB**, **Keefe CR**, **Keim P**, **Kelley ST**, **Knights D**, **Koester I**, **Kosciolek T**, **Kreps J**, **Langille MGI**, **Lee J**, **Ley R**, **Liu Y-X**, **Loftfield E**, **Lozupone C**, **Maher M**, **Marotz C**, **Martin BD**, **McDonald D**, **McIver LJ**, **Melnik AV**, **Metcalf JL**, **Morgan SC**, **Morton JT**, **Naimey AT**, **Navas-Molina JA**, **Nothias LF**, **Orchanian SB**, **Pearson T**, **Peoples SL**, **Petras D**, **Preuss ML**, **Pruesse E**, **Rasmussen LB**, **Rivers A**, **Robeson MS**, **Rosenthal P**, **Segata N**, **Shaffer M**, **Shiffer A**, **Sinha R**, **Song SJ**, **Spear JR**, **Swafford AD**, **Thompson LR**, **Torres PJ**, **Trinh P**, **Tripathi A**, **Turnbaugh PJ**, **Ul-Hasan S**, **van der Hooft JJJ**, **Vargas F**, **Vázquez-Baeza Y**, **Vogtmann E**, **von Hippel M**, **Walters W**, **Wan Y**, **Wang M**, **Warren J**, **Weber KC**, **Williamson CHD**, **Willis AD**, **Xu ZZ**, **Zaneveld JR**, **Zhang Y**, **Zhu Q**, **Knight R**, **Caporaso JG**. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol **37**:852–857.
384   doi:10.1038/s41587-019-0209-9.

385   19.   **Köster J**, **Rahmann S**. 2012. Snakemake — a scalable bioinformatics workflow
386   engine. Bioinformatics **28**:2520–2522. doi:10.1093/bioinformatics/bts480.

387   20.   **R Core Team**. 2020. R: A language and environment for statistical computing.
388   Manual, R Foundation for Statistical Computing, Vienna, Austria.

389   21.   **Van Rossum G**, **Drake FL**. 2009. Python 3 Reference Manual | Guide books.
390

22. Bash Reference Manual. https://www.gnu.org/software/bash/manual/bash.html.

23. **Schloss PD**, **Westcott SL**, **Ryabin T**, **Hall JR**, **Hartmann M**, **Hollister EB**, **Lesniewski RA**, **Oakley BB**, **Parks DH**, **Robinson CJ**, **Sahl JW**, **Stres B**, **Thallinger GG**, **Van Horn DJ**, **Weber CF**. 2009. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology **75**:7537–7541. doi:10.1128/AEM.01541-09.

24. **Wickham H**, **Averick M**, **Bryan J**, **Chang W**, **McGowan LD**, **François R**, **Grolemund G**, **Hayes A**, **Henry L**, **Hester J**, **Kuhn M**, **Pedersen TL**, **Miller E**, **Bache SM**, **Müller K**, **Ooms J**, **Robinson D**, **Seidel DP**, **Spinu V**, **Takahashi K**, **Vaughan D**, **Wilke C**, **Woo K**, **Yutani H**. 2019. Welcome to the Tidyverse. Journal of Open Source Software **4**:1686. doi:10.21105/joss.01686.

25. **Xie Y**, **Allaire JJ**, **Grolemund G**. 2018. R Markdown: The Definitive Guide. Taylor & Francis, CRC Press.

26. **Pedersen TL**. 2021. Ggraph: An implementation of grammar of graphics for graphs and networks.

27. **Wilke CO**. 2020. Ggtext: Improved text rendering support for 'Ggplot2'. Manual.

28. **Harris CR**, **Millman KJ**, **van der Walt SJ**, **Gommers R**, **Virtanen P**, **Cournapeau D**, **Wieser E**, **Taylor J**, **Berg S**, **Smith NJ**, **Kern R**, **Picus M**, **Hoyer S**, **van Kerkwijk MH**, **Brett M**, **Haldane A**, **del Río JF**, **Wiebe M**, **Peterson P**, **Gérard-Marchant P**, **Sheppard K**, **Reddy T**, **Weckesser W**, **Abbasi H**, **Gohlke C**, **Oliphant TE**. 2020. Array programming with NumPy. Nature **585**:357–362. doi:10.1038/s41586-020-2649-2.

405   29.   SRA-Tools - NCBI. http://ncbi.github.io/sra-tools/.

406

407   30.   2016. Anaconda Software Distribution. Anaconda Documentation. Anaconda Inc.

408