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

2022-01-13

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

Abstract

Assigning amplicon sequences to operational taxonomic units (OTUs) is an important step in characterizing microbial communities across large datasets. 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. However, one may wish to incorporate new samples to previously clustered datasets without clustering all sequences again, such as when comparing across datasets or deploying machine learning models. Existing reference-based methods produce consistent OTUs, but only consider the similarity of each query sequence to a single reference sequence in an OTU, resulting in assignments that are worse than those generated by *de novo* methods. To provide an efficient method to fit sequences to existing OTUs, we developed the OptiFit algorithm. Inspired by the *de novo* OptiClust algorithm, OptiFit considers the similarity of all pairs of reference and query sequences to produce OTUs of the best possible quality. We tested OptiFit using four datasets with two strategies: 1) clustering to a reference database or 2) splitting the dataset into a reference and query set, clustering the references using OptiClust, then clustering the queries to the references. The result is an improved implementation of reference-based clustering. OptiFit produces similar quality OTUs as OptiClust at faster speeds when using the split dataset strategy. OptiFit provides a suitable option for users requiring 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

27 assignments can change when new sequences are added to a dataset, making it difficult

28 to compare different studies. Other approaches assign sequences to groups by comparing

29 them to sequences in a reference database to produce consistent assignments, but the

30 quality of the groups produced is reduced compared to OptiClust. We developed OptiFit, a

31 new reference-based algorithm that produces consistent yet high quality assignments like

32 OptiClust. OptiFit allows researchers to compare microbial communities across different

33 studies or add new data to existing studies without sacrificing the quality of the group

34 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). OTU quality can be conceptualized as how well the OTU assignments match the definition set by the distance threshold, i.e. whether sequence pairs that are at least as similar as the distance threshold are assigned to the same OTU and sequence pairs that are more dissimilar than the distance threshold are assigned to different OTUs.

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

₆₁ positives.

₆₂ A limitation of *de novo* clustering is that different OTU assignments will be produced
₆₃ when new sequences are added to a dataset, making it difficult to use *de novo* clustering
₆₄ to compare OTUs between different studies. Furthermore, since *de novo* clustering
₆₅ requires calculating and comparing distances between all sequences in a dataset, the
₆₆ execution time can be slow and memory requirements can be prohibitive for very large
₆₇ datasets. Reference clustering attempts to overcome the limitations of *de novo* clustering
₆₈ methods by using a representative set of sequences from a database, with each reference
₆₉ sequence seeding an OTU. Commonly, the Greengenes set of representative full length
₇₀ sequences clustered at 97% similarity is used as the reference with VSEARCH (5–7).
₇₁ Query sequences are then clustered into OTUs based on their similarity to the reference
₇₂ sequences. Any query sequences that are not within the distance threshold to any of
₇₃ the reference sequences are either thrown out (closed reference clustering) or clustered
₇₄ *de novo* to create additional OTUs (open reference clustering). While reference-based
₇₅ clustering is generally fast, it is limited by the diversity of the reference database. Novel
₇₆ sequences in the sample will be lost in closed reference mode if they are not represented
₇₇ by a similar sequence in the database. We previously found that the OptiClust *de novo*
₇₈ clustering algorithm created the highest quality OTU assignments of all clustering methods
₇₉ (1).

₈₀ To overcome the limitations of current reference-based and *de novo* clustering algorithms
₈₁ while maintaining OTU quality, we developed OptiFit, a reference-based clustering
₈₂ algorithm. While other tools represent reference OTUs with a single sequence, OptiFit
₈₃ uses all sequences in existing OTUs as the reference and fits new sequences to those
₈₄ reference OTUs. In contrast to other tools, OptiFit considers all pairwise distance scores
₈₅ between reference and query sequences when assigning sequences to OTUs in order to
₈₆ produce OTUs of the highest possible quality. Here, we tested the OptiFit algorithm with

87 the reference as a public database (e.g. Greengenes) or *de novo* OTUs generated using a

88 reference set from the full dataset and compared the performance to existing tools. To

89 evaluate the OptiFit algorithm and compare to existing methods, we used four published

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

91 OptiFit is available within the mothur software program.

Results

The OptiFit algorithm

94 OptiFit leverages the method employed by OptiClust of iteratively assigning sequences

95 to OTUs to produce the highest quality OTUs possible, and extends this method for

96 reference-based clustering. OptiClust first seeds each sequence into its own OTU as a

97 singleton. Then for each sequence, OptiClust considers whether the sequence should

98 move to a different OTU or remain in its current OTU, choosing the option that results in

99 a better MCC score (1). The MCC uses all values from a confusion matrix and ranges

100 from negative one to one, with a score of one occurring when all sequence pairs are true

101 positives and true negatives, a score of negative one occurring when all pairs are false

102 positives and false negatives, and a score of zero when there are equal numbers of true

103 and false assignments (i.e. no better than random guessing). Sequence pairs that are

104 similar to each other (i.e. within the distance threshold) are counted as true positives if

105 they are clustered into the same OTU, and false negatives if they are not in the the same

106 OTU. Sequence pairs that are not similar to each other are true negatives if they are not

107 clustered into the same OTU, and false positives if they are not in the same OTU. Thus, a

108 pair of sequences is considered correctly assigned when their OTU assignment matches

109 the OTU definition set by the distance threshold. OptiClust iterations continue until the

110 MCC stabilizes or until a maximum number of iterations is reached. This process produces

111 *de novo* OTU assignments with the most optimal MCC given the input sequences.

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

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 of 3%. Previously, 50 reference sequences were clustered *de novo* with OptiClust (see the OptiClust supplemental text (1)). Reference sequences A through Q (colored orange) were within the distance threshold to at least one other reference sequence; the remaining reference sequences formed additional singleton OTUs (not shown). The goal of OptiFit is to assign the query sequences W through Z (colored green) to the reference OTUs. Here, there are 50 reference sequences and 4 query sequences which make 1,431 sequence pairs, of which 23 pairs are within the 3% distance threshold. Initially (step 1), OptiFit places each query sequence in its own OTU, resulting in 14 true positives, 9 false negatives, 0 false positives, and 1,408 true negatives for an MCC score of 0.78. 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 (steps 2-4). 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 was needed. Note that sequence Z was dissimilar from all other sequences and thus it remained a singleton. The final MCC score is 0.91 with 20 true positives, 3 false negatives, 1 false positive, and 1407 true negatives.

112 OptiFit begins where OptiClust ends, starting with a list of reference OTUs and their

113 sequences, a list of query sequences to cluster to the reference OTUs, and the sequence

114 pairs that are within the distance threshold (e.g. 0.03) (Figure 1). Initially, all query

115 sequences are placed into separate OTUs. Then, the algorithm iteratively reassigns the

116 query sequences to the reference OTUs to optimize the MCC. Alternatively, a sequence

117 will remain unassigned if the MCC value is maximized when the sequence is a singleton

118 rather than clustered into a reference OTU. All query and reference sequence pairs are

119 considered when calculating the MCC. This process is repeated until the MCC changes by

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

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

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

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

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

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

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

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

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

129 **Reference clustering with public databases**

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

131 to three databases of reference OTUs: the Greengenes database v13_8_99 (6), the

132 SILVA non-redundant database v132 (12), and the Ribosomal Database Project (RDP) v16

133 (13). Reference OTUs for each database were created by performing *de novo* clustering

134 with OptiClust at a distance threshold of 3% using the V4 region of each sequence (see

135 Figure 2). After trimming to the V4 region, the databases contained 174,979, 16,192, and

136 173,648 unique sequences and produced *de novo* MCC scores of 0.72, 0.74, and 0.73 for

137 Greengenes, RDP, and SILVA, respectively. Clustering query sequences with OptiFit to

138 Greengenes and SILVA in closed reference mode performed similarly, with median MCC

139 scores of 0.85 and 0.77 respectively, while the median MCC was 0.35 when clustering to

140 RDP (Figure 3; "db: Greengenes", "db: SILVA", and "db: RDP"). For comparison, clustering

141 datasets with OptiClust produced an average MCC score of 0.86 (Figure 3; "*de novo*").

142 This gap in OTU quality mostly disappeared when clustering in open reference mode,

143 which produced median MCCs of 0.86 with Greengenes, 0.86 with SILVA, and 0.86 with

144 the RDP. Thus, open reference OptiFit produced OTUs of very similar quality as *de novo*

145 clustering with OptiClust, and closed reference OptiFit followed closely behind as long as a

146 suitable reference database was chosen.

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

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

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

150 sequences were clustered with Greengenes as the reference (59%) than with SILVA (50%)

151 or with the RDP (9.7%) (Figure 3). This mirrored the result reported above that Greengenes

152 produced better OTUs in terms of MCC score than either SILVA or RDP. Note that *de novo*

153 and open reference clustering methods always cluster 100% of sequences into OTUs.

154 The database chosen affects the final closed reference OTU assignments considerably in

155 terms of both MCC score and fraction of query sequences that could be clustered into the

156 reference OTUs.

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

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

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

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

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

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

163 406.8 for closed reference OptiFit, 455.3 for *de novo* clustering the dataset, and 559.4 for
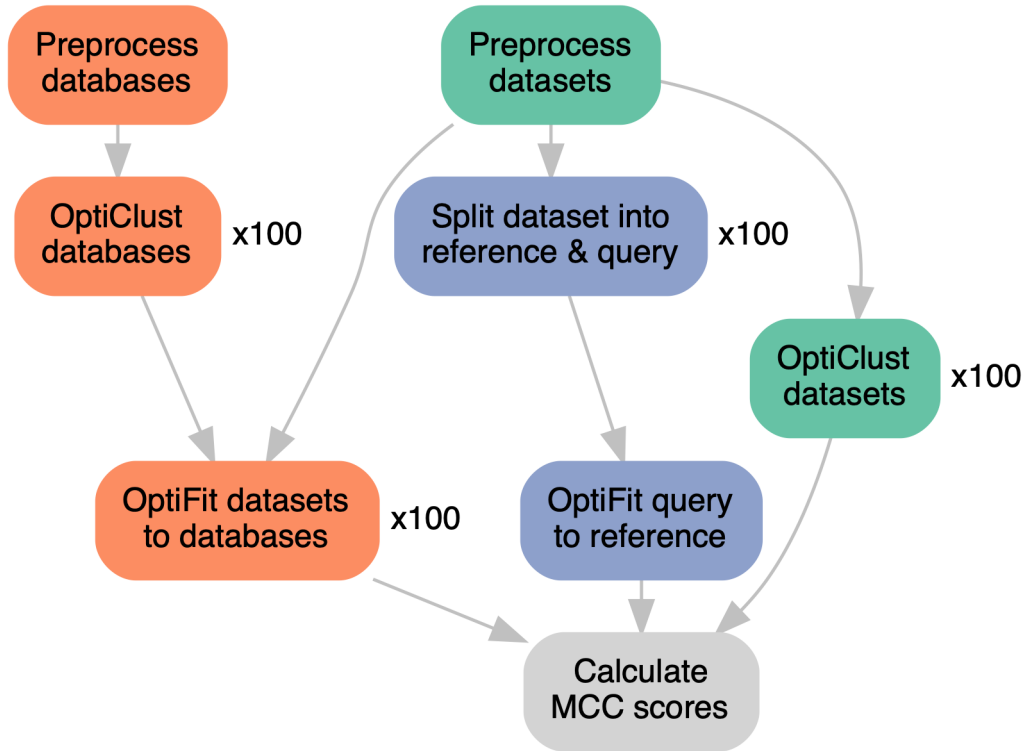
Figure 2: The Analysis Workflow. Reference sequences from Greengenes, the RDP, and SILVA were downloaded, preprocessed with mothur by trimming to the V4 region, and clustered *de novo* with OptiClust for 100 repetitions. Datasets from human, marine, mouse, and soil microbiomes were downloaded, preprocessed 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.

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

165 reference clustering sacrifices OTU quality for execution speed.

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

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

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

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

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

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

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

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

174 higher quality OTUs than VSEARCH in terms of MCC score, but VSEARCH was able to

175 cluster 24.9% more query sequences than OptiFit to the Greengenes reference database

176 (Figure 3). This is because VSEARCH only considers the distances between each query

177 sequence to the single reference sequence, while OptiFit considers the distances between

178 all pairs of reference and query sequences in an OTU. When open reference clustering,

179 OptiFit produced higher quality OTUs than VSEARCH against the Greengenes database,

180 with median MCC scores of 0.86 and 0.56, respectively. In terms of run time, OptiFit

181 outperformed VSEARCH in both closed and open reference mode by 53.6% and 44.0% on

182 average, respectively. Thus, the more stringent OTU definition employed by OptiFit, which

183 prefers the query sequence to be similar to all other sequences in the OTU rather than to

184 only one sequence, resulted in fewer sequences being clustered to reference OTUs than

185 when using VSEARCH, but caused OptiFit to outperform VSEARCH in terms of both OTU

186 quality and execution time.
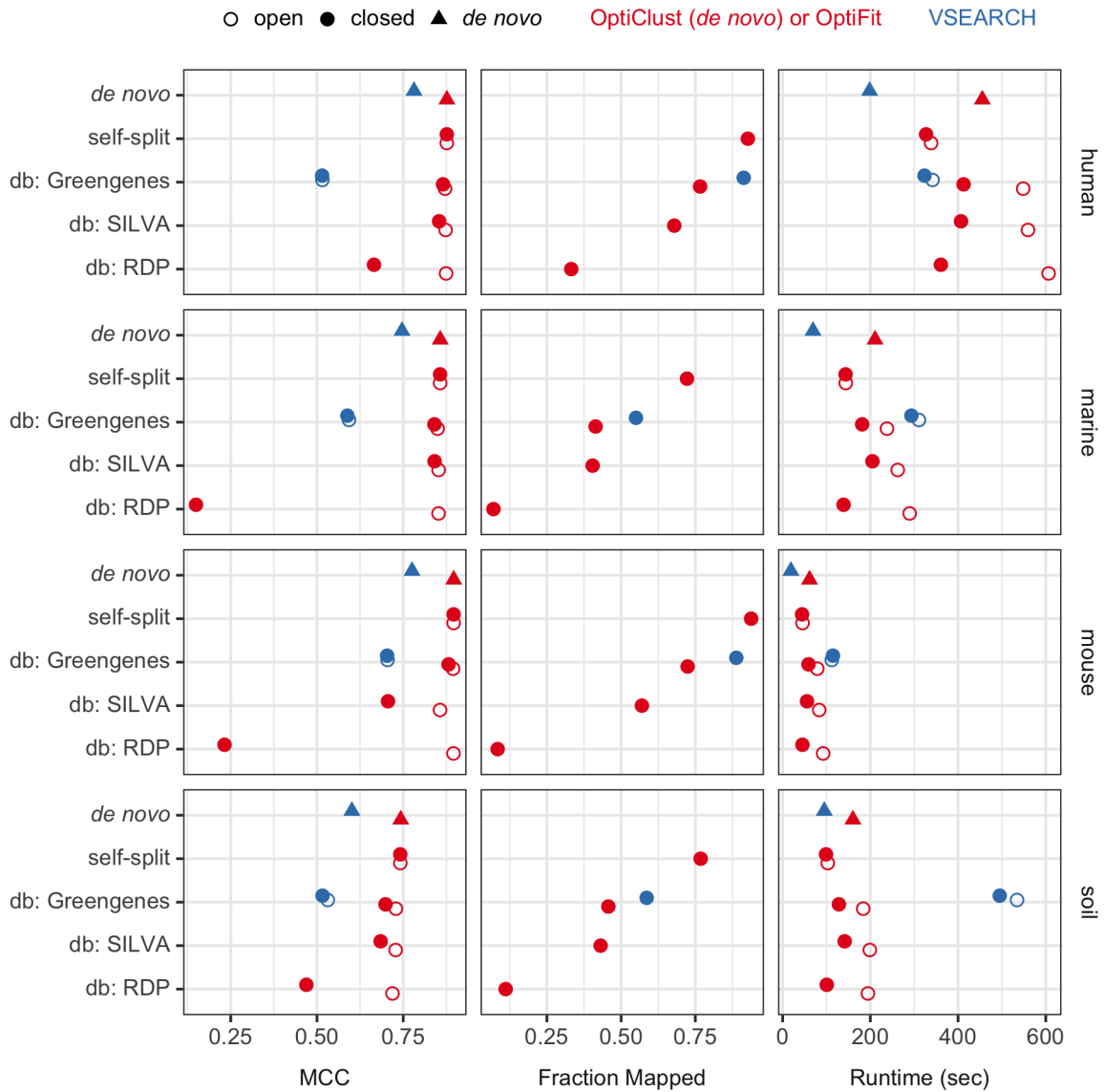
Figure 3: Benchmarking Results. The median MCC score, fraction of query sequences that mapped in closed-reference clustering, and runtime in seconds from repeating each clustering method 100 times. Each dataset underwent three clustering strategies; 1) *de novo* clustering the whole dataset using OptiClust, 2) splitting the dataset with 50% of the sequences as a reference set and the other 50% as a query set, clustering the references using OptiClust, then clustering the query sequences to the reference OTUs with OptiFit, or 3) clustering the dataset to a reference database. 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.
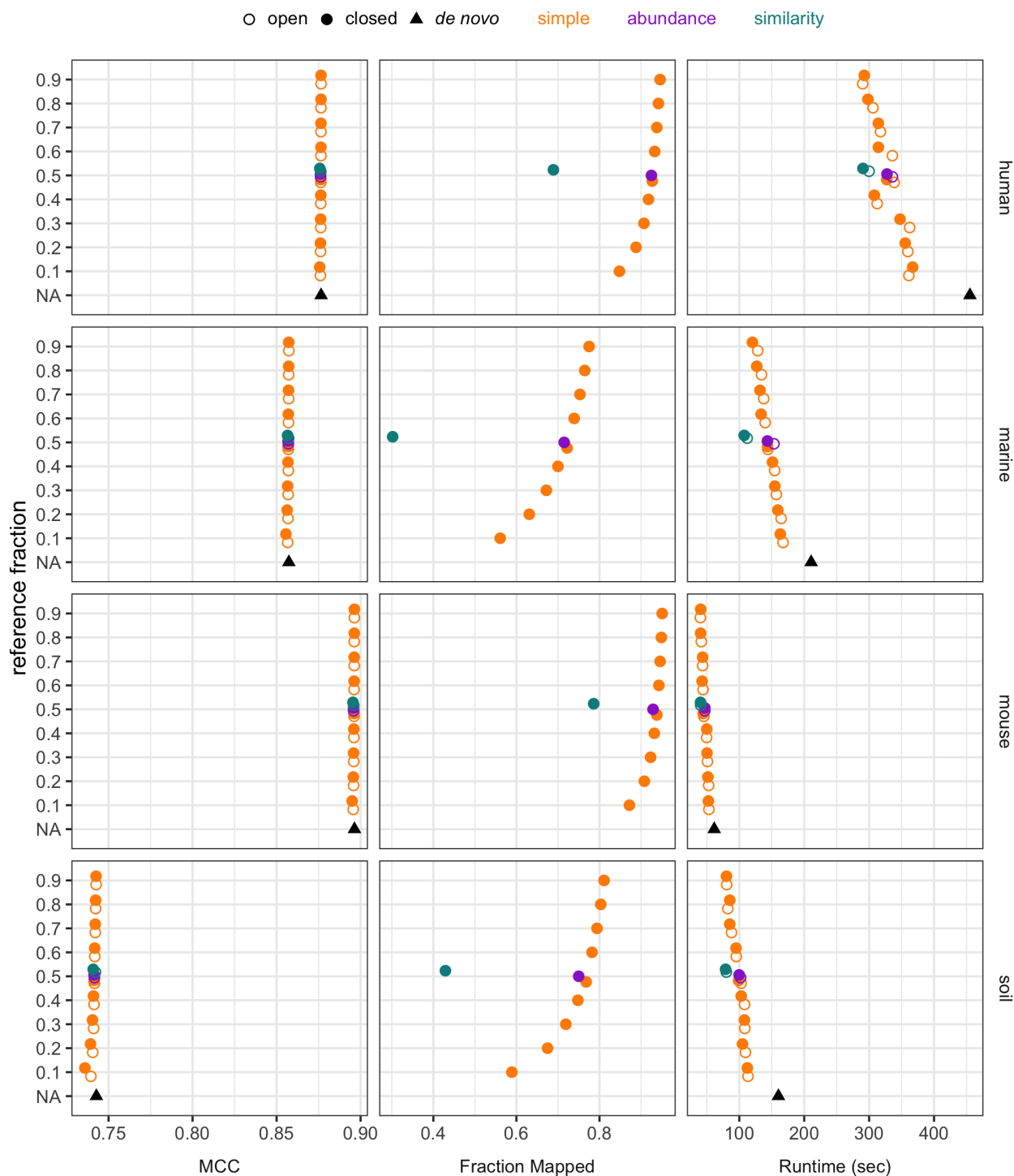
Reference clustering with split datasets

When performing reference clustering against public databases, the database chosen greatly affects the quality of OTUs produced. OTU quality may be poor when the reference database consists of sequences that are too unrelated to the samples of interest, such as when samples contain novel populations. While *de novo* clustering overcomes the quality limitations of reference clustering to databases, OTU assignments are not consistent when new sequences are added. Researchers may wish to cluster new sequences to existing OTUs or to compare OTUs across studies. To determine how well OptiFit performs for clustering new sequences to existing OTUs, we employed a split dataset strategy, where each dataset was randomly split into a reference fraction and a query fraction. Reference sequences were clustered *de novo* with OptiClust, then query sequences were clustered 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 (0.031% difference in median MCC). In closed reference mode, OptiFit was able to cluster 84.9% of query sequences to reference OTUs with the split strategy, a great improvement over the average 59% 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 39.6% and 36.8%, respectively. Random access memory (RAM) usage was similar, with OptiFit requiring slightly more RAM in gigabytes than OptiClust. Open and closed reference OptiFit required 1.8% and 1.2% more RAM than OptiClust, respectively (data not shown). The split dataset strategy also performed 6.7% faster than the database strategy in closed reference mode and 65.5% 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

213 more query sequences than the database strategy.

214 While we initially tested this strategy using a 50% split of the data into reference and query

215 fractions, we next investigated whether there was an optimal reference fraction size. To

216 identify the best reference size, reference sets with 10% to 90% of the sequences were

217 created, with the remaining sequences used for the query (Figure 4). OTU quality was

218 remarkably consistent across reference fraction sizes. For example, splitting the human

219 dataset 100 times yielded a coefficient of variation (i.e. the standard deviation divided by

220 the mean) of 0.0018 for the MCC score across all fractions. Run time generally decreased

221 as the reference fraction increased; for the human dataset, the median run time was

222 364.0 seconds with 10% of sequences in the reference and 290.8 seconds with 90% of

223 sequences in the reference. The RAM usage was virtually the same across reference

224 fraction sizes, with a coefficient of variation of 0.00089 for the human dataset (data not

225 shown). In closed reference mode, the fraction of sequences that mapped increased as

226 the reference size increased; for the human dataset, the median fraction mapped was 0.85

227 with 10% of sequences in the reference and 0.95 with 90% of sequences in the reference.

228 These trends held for the other datasets as well. Thus, the reference fraction did not affect

229 OTU quality in terms of MCC score nor the memory usage, but did affect the run time and

230 the fraction of sequences that mapped during the closed reference clustering.

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

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

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

234 50%: a simple random sample, weighting sequences by relative abundance, and weighting

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

236 was similar across all three sampling methods (median MCC of 0.86). In closed-reference

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

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

14

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 with OptiClust is also shown for comparison.

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

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

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

242 103.9 seconds compared to 135.4 and 134.8 seconds for simple and abundance-weighted

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

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

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

246 the case of similarity-weighted sampling.

Discussion

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

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

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

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

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

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

254 for tasks that do not require reference OTUs.

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

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

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

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

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

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

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

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

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

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

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

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

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

268   OTUs are not required.

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

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

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

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

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

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

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

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

277   each query sequence to be similar to the single centroid sequence that seeded the OTU,

278   thus allowing pairs of query sequences to be less similar to each other than the threshold

279   specified. Because of this, VSEARCH sacrifices OTU quality by allowing more dissimilar

280   sequences to be clustered into the same OTUs.

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

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

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

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

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

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

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

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

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

17

290   both run time and OTU quality.

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

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

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

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

295   sequences to reference databases, comparing taxonomic composition of microbiomes

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

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

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

Materials and Methods

Data Processing Steps

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

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

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

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

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

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

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

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

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

Reference database clustering

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

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

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

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

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

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

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

318   97% OTU reference alignment, since this method is how VSEARCH is typically used by

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

Split dataset clustering

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

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

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

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

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

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

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

328   reference OTUs with OptiFit.

Benchmarking

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

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

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

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

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

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

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

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

Data and Code Availability

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

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

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

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

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

Sovacool_OptiFit_mSphere_2022.

Acknowledgements

Author Contributions

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

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

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

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

reviewed and edited the manuscript.

References

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

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

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

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

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

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. doi:10.1128/AEM.03006-05.

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

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 Alaska Tundra Ecosystem. Front Microbiol **7**. doi:10.3389/fmicb.2016.00579.

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

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

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

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. doi:10.1093/nar/gks1219.

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. doi:10.1093/nar/gkt1244.

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

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

388   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.
389   Appl Environ Microbiol **79**:5112–5120. doi:10.1128/AEM.01043-13.

390   17.   **Edgar RC**, **Haas BJ**, **Clemente JC**, **Quince C**, **Knight R**. 2011.   UCHIME
improves sensitivity and speed of chimera detection. Bioinformatics **27**:2194–2200.
391   doi:10.1093/bioinformatics/btr381.

392   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.
doi:10.1038/s41587-019-0209-9.

393

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

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

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

399

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

401

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.

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

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