OptiClust: Improved method for assigning amplicon-based sequence data to operational taxonomic units

Running title: OptiClust: Optimized Clustering

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Abstract

Assignment of 16S rRNA gene sequences to operational taxonomic units (OTUs) is a computational bottleneck in the process of analyzing microbial communities. Although this has been an active area of research, it has been difficult to overcome the time and memory demands while improving the quality of the OTU assignments. Here we developed a new OTU assignment algorithm that iteratively reassigns sequences to new OTUs to optimize the Matthews correlation coefficient (MCC), a measure of the quality of OTU assignments. To assess the new algorithm, OptiClust, we compared it to ten other algorithms using 16S rRNA gene sequences from two simulated and four natural communities. Using the OptiClust algorithm, the MCC values averaged 15.2 and 16.5% higher than the OTUs generated when we used the average neighbor and distance-based greedy clustering with VSEARCH, respectively. Furthermore, on average, OptiClust was 94.6-times faster than the average neighbor algorithm and just as fast as distance-based greedy clustering with VSEARCH. An empirical analysis of the efficiency of the algorithms showed that the time and memory required to perform the algorithm scaled quadratically with the number of unique sequences in the dataset. The significant improvement in the quality of the OTU assignments over previously existing methods will significantly enhance downstream analysis by limiting the splitting of similar sequences into separate OTUs and merging of dissimilar sequences into the same OTU. The development of the OptiClust algorithm represents a significant advance that is likely to have numerous other applications.

Importance

The analysis of microbial communities from diverse environments using 16S rRNA gene sequencing has expanded our knowledge of the biogeography of microorganisms. An important step in this analysis is the assignment of sequences into taxonomic groups based on their similarity to sequences in a database or based on their similarity to each other, irrespective of a database. In this study, we present a new algorithm for the latter approach. The algorithm, OptiClust, seeks to optimize a metric of assignment quality by shuffling sequences between taxonomic groups.

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27 We found that OptiClust produces more robust assignments and does so in a rapid and memory

28 efficient manner. This advance will allow for a more robust analysis of microbial communities and

29 the factors that shape them.

Introduction

Amplicon-based sequencing has provided incredible insights into Earth's microbial biodiversity (1, 2). It has become common for studies to include sequencing millions of 16S rRNA gene sequences across hundreds of samples (3, 4). This is three to four orders of magnitude greater sequencing depth than was previously achieved using Sanger sequencing (5, 6). The increased sequencing depth has revealed novel taxonomic diversity that is not adequately represented in reference databases (1, 3). However, the advance has forced re-engineering of methods to overcome the rate and memory limiting steps in computational pipelines that process raw sequences through the generation of tables containing the number of sequences in different taxa for each sample (7–10). A critical component to these pipelines has been the assignment of amplicon sequences to taxonomic units that are ether defined based on similarity to a reference or operationally based on the similarity of the sequences to each other within the dataset (11, 12).

A growing number of algorithms have been developed to cluster sequences into OTUs. These algorithms can be classified into three general categories. The first category of algorithms has been termed closed-reference or phylotyping (13, 14). Sequences are compared to a reference collection and clustered based on the reference sequences that they are similar to. This approach is fast; however, the method struggles when a sequence is similar to multiple reference sequences that may have different taxonomies and when it is not similar to sequences in the reference (15). The second category of algorithms has been called *de novo* because they assign sequences to OTUs without the use of a reference (14). These include hierarchical algorithms such as nearest, furthest, and average neighbor (16) and algorithms that employ heuristics such as abundance or distance-based greedy clustering as implemented in USEARCH (17) or VSEARCH (18), Sumaclust, OTUCLUST (19), and Swarm (20). *De novo* methods are agglomerative and tend to be more computationally intense. It has proven difficult to know which method generates the best assignments. A third category of algorithm is open reference clustering, which is a hybrid approach (3, 14). Here sequences are assigned to OTUs using closed-reference clustering and sequences that are not within a threshold of a reference sequence are then clustered using a *de novo* approach. This category blends the strengths and weaknesses of the other method and adds the complication that

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58 closed-reference and *de novo* clustering use different OTU definitions. These three categories of

59 algorithms take different approaches to handling large datasets to minimize the time and memory

60 requirements while attempting to assign sequences to meaningful OTUs.

61 Several metrics have emerged for assessing the quality of OTU assignment algorithms. These have

62 included the time and memory required to run the algorithm (3, 20–22), agreement between OTU

63 assignments and the sequences' taxonomy (20, 22–32), sensitivity of an algorithm to stochastic

64 processes (33), the number of OTUs generated by the algorithm (23, 34), and the ability to

65 regenerate the assignments made by other algorithms (3, 35). Unfortunately, these methods fail

66 to directly quantify the quality of the OTU assignments. An algorithm may complete with minimal

67 time and memory requirements or generate an idealized number of OTUs, but the composition of

68 the OTUs could be incorrect. These metrics also tend to be subjective. For instance, a method

69 may appear to recapitulate the taxonomy of a synthetic community with known taxonomic structure,

70 but do a poor job when applied to real communities with poorly defined taxonomic structure or for

71 sequences that are prone to misclassification. As an alternative, we developed an approach to

72 objectively benchmark the clustering quality of OTU assignments (13, 15, 36). This approach counts

73 the number of true positives (TP), true negatives (TN), false positives (FP), and false negatives

74 (FN) based on the pairwise distances. Sequence pairs that are within the user-specified threshold

75 and are clustered together represent TPs and those in different OTUs are FNs. Those sequence

76 pairs that have a distance larger than the threshold and are not clustered in the same OTU are TNs

77 and those in the same OTU are FPs. These values can be synthesized into a single correlation

78 coefficient, the Matthews correlation coefficient (MCC), which measures the correlation between

79 observed and predicted classifications and is robust to cases where there is an uneven distribution

80 across the confusion matrix (37). Consistently, the average neighbor algorithm was identified as

81 among the best or the best algorithm. Other hierarchical algorithms such as furthest and nearest

82 neighbor, which do not permit the formation of FPs or FNs, respectively, fared significantly worse.

83 The distance-based greedy clustering as implemented in VSEARCH has also performed well. The

84 computational resources required to complete the average neighbor algorithm can be significant for

85 large datasets and so there is a need for an algorithm that efficiently produces consistently high

86 quality OTU assignments.

87 These benchmarking efforts have assessed the quality of the clusters after the completion of the

88 algorithm. In the current study we developed and benchmarked a new *de novo* clustering algorithm

89 that uses real time calculation of the MCC to direct the progress of the clustering. The result is

90 the OptiClust algorithm, which produces significantly better sequence assignments while making

91 efficient use of computational resources.

Results

93 ***OptiClust algorithm.*** The OptiClust algorithm uses the pairs of sequences that are within a desired

94 threshold of each other (e.g. 0.03), a list of all sequence names in the dataset, and the metric that

95 should be used to assess clustering quality. A detailed description of the algorithm is provided for a

96 toy dataset in the Supplementary Material. Briefly, the algorithm starts by placing each sequence

97 either within its own OTU or into a single OTU. The algorithm proceeds by interrogating each

98 sequence and re-calculating the metric for the cases where the sequence stays in its current OTU,

99 is moved to each of the other OTUs, or is moved into a new OTU. The location that results in the

100 best clustering quality indicates whether the sequence should remain in its current OTU or be

101 moved to a different or new OTU. Each iteration consists of interrogating every sequence in the

102 dataset. Although numerous options are available for optimizing the clusters and for assessing

103 the quality of the clusters within the mothur-based implementation of the algorithm (e.g. sensitivity,

104 specificity, accuracy, F1-score, etc.), the default metric for optimization and assessment is MCC

105 because it includes all four parameters from the confusion matrix (Figure S1; Table S1). The

106 algorithm continues until the optimization metric stabilizes or until it reaches a defined stopping

107 criteria.

108 ***OptiClust-generated OTUs are more robust than those from other methods.*** To evaluate the

109 OptiClust algorithm and compare its performance to other algorithms, we utilized six datasets

110 including two synthetic communities and four previously published large datasets generated from

111 soil, marine, human, and murine samples (Table 1). When we seeded the OptiClust algorithm with

112 each sequence in a separate OTU and ran the algorithm until complete convergence, the MCC

113 values averaged 15.2 and 16.5% higher than the OTUs using average neighbor and distance-based

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114 greedy clustering (DGC) with VSEARCH, respectively (Figure 1; Table S1). The number of OTUs

115 formed by the various methods was negatively correlated with their MCC value (ρ=-0.47; p<0.001).

116 The OptiClust algorithm was considerably faster than the hierarchical algorithms and somewhat

117 slower than the heuristic-based algorithms. Across the six datasets, the OptiClust algorithm was

118 94.6-times faster than average neighbor and just as fast as DGC with VSEARCH. The human

119 dataset was a challenge for a number of the algorithms. OTUCLUST and SumaClust were unable

120 to cluster the human dataset in less than 50 hours and the average neighbor algorithm required

121 more than 45 GB of RAM. The USEARCH-based methods were unable to cluster the human data

122 using the 32-bit free version of the software that limits the amount of RAM to approximately 3.5 GB.

123 These data demonstrate that OptiClust generated significantly more robust OTU assignments than

124 existing methods across a diverse collection of datasets with performance that was comparable to

125 popular methods.

126 **_OptiClust stopping criteria._** By default, the mothur-based implementation of the algorithm stops

127 when the optimization metric changes by less than 0.0001; however, this can be altered by the user.

128 This implementation also allows the user to stop the algorithm if a maximum number of iterations is

129 exceeded. By default mothur uses a maximum value of 100 iterations. The justification for allowing

130 incomplete convergence was based on the observation that numerous iterations are performed

131 that extend the time required to complete the clustering with minimal improvement in clustering

132 (Figure S2). We evaluated the results of clustering to partial convergence (i.e. a change in the

133 MCC value that was less than 0.0001) or until complete convergence of the MCC value (i.e. until it

134 did not change between iterations) when seeding the algorithm with each sequence in a separate

135 OTU (Figure 1). The small difference in MCC values between the output from partial and complete

136 convergence resulted in a difference in the median number of OTUs that ranged between 1.5 and

137 17.0 OTUs. This represented a difference of less than 0.15%. Among the four natural datasets,

138 between 3 and 6 were needed to achieve partial convergence and between 8 and 12 iterations

139 were needed to reach full convergence. The additional steps required between 1.4 and 1.7 times

140 longer to complete the algorithm. These results suggest that achieving full convergence of the

141 optimization metric adds computational effort; however, considering full convergence took between

142 2 and 17 minutes the extra effort was relatively small. Although the mothur's default setting is partial

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143 convergence, the remainder of our analysis used complete convergence to be more conservative.

144 **_Effect of seeding OTUs on OptiClust performance._** By default the mothur implementation of

145 the OptiClust algorithm starts with each sequence in a separate OTU. An alternative approach

146 is to start with all of the sequences in a single OTU. We found that the MCC values for clusters

147 generated seeding OptiClust with the sequences as a single OTU were between 0 and 11.5% lower

148 than when seeding the algorithm with sequences in separate OTUs (Figure 1). Interestingly, with

149 the exception of the human dataset (0.2% more OTUs), the number of OTUs was as much as 7.0%

150 lower (mice) than when the algorithm was seeded with sequence in separate OTUs. Finally, the

151 amount of time required to cluster the data when the algorithm was seeded with a single OTU was

152 between 1.5 and 2.9-times longer than if sequences were seeded as separate OTUs. This analysis

153 demonstrates that seeding the algorithm with sequences as separate OTUs resulted in the best

154 OTU assignments in the shortest amount of time.

155 **_OptiClust-generated OTUs are as stable as those from other algorithms._** One concern that

156 many have with _de novo_ clustering algorithms is that their output is sensitive to the initial order of

157 the sequences because each algorithm must break ties where a sequence could be assigned to

158 multiple OTUs. An additional concern specific to the OptiClust algorithm is that it may stabilize

159 at a local optimum. To evaluate these concerns we compared the results obtained using ten

160 randomizations of the order that sequences were given to the algorithm. The median coefficient

161 of variation across the six datasets for MCC values obtained from the replicate clusterings using

162 OptiClust was 0.1% (Figure 1). We also measured the coefficient of variation for the number of

163 OTUs across the six datasets for each method. The median coefficient of variation for the number of

164 OTUs generated using OptiClust was 0.1%. Confirming our previous results (15), all of the methods

165 we tested were stable to stochastic processes. Of the methods that involved randomization, the

166 coefficient of variation for MCC values was considerably smaller with OptiClust than the other

167 methods and the coefficient of variation for the number of OTUs was comparable to the other

168 methods. The variation observed in clustering quality suggested that the algorithm does not appear

169 to converge to a locally optimum MCC value. More importantly, the random variation does yield

170 output of a similarly high quality.

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Time and memory required to complete Optimization-based clustering scales efficiently.
Although not as important as the quality of clustering, the amount of time and memory required to assign sequences to OTUs is a legitimate concern. We observed that the time required to complete the OptiClust algorithm (Figure 1C) paralleled the number of pairwise distances that were smaller than 0.03 (Table 1). To further evaluate how the speed and memory usage scaled with the number of sequences in the dataset, we measured the time required and maximum RAM usage to cluster 20, 40, 60, 80, and 100% of the unique sequences from each of the natural datasets using the OptiClust algorithm (Figure 2). Within each iteration of the algorithm, each sequence is compared to every other sequence and each comparison requires a recalculation of the confusion matrix. This would result in a worst case algorithmic complexity on the order of N^3, where N is the number of unique sequences. Because the algorithm only needs to keep track of the sequence pairs that are within the threshold of each other, it is likely that the implementation of the algorithm is more efficient. To empirically determine the algorithmic complexity, we fit a power law function to the data in Figure 2A. We observed power coefficients between 1.7 and 2.5 for the marine and human datasets, respectively. The algorithm requires storing a matrix that contains the pairs of sequences that are close to each other as well as a matrix that indicates which sequences are clustered together. The memory required to store these matrices is on the order of N^2, where N is the number of unique sequences. In fact, when we fit a power law function to the data in Figure 2B, the power coefficients were 1.9. Using the four natural community datasets, doubling the number of sequences in a dataset would increase the time required to cluster the data by 4 to 8-fold and increase the RAM required by 4-fold. It is possible that future improvements to the implementation of the algorithm could improve this performance.

Cluster splitting heuristic generates OTUs that are as good as non-split approach. We previously described a heuristic to accelerate OTU assignments where sequences were first classified to taxonomic groups and within each taxon sequences were assigned to OTUs using the average neighbor clustering algorithm (13). This method is similar to open reference clustering except that in our approach all sequences are subjected to *de novo* clustering following classification whereas in open reference clustering only those sequences that cannot be classified are subjected to *de novo* clustering. Our cluster splitting approach accelerated the clustering and reduced the

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200 memory requirements because the number of unique sequences was effectively reduced by splitting

201 sequences across taxonomic groups. Furthermore, because sequences in different taxonomic

202 groups are assumed to belong to different OTUs they are independent, which permits parallelization

203 and additional reduction in computation time. Reduction in clustering quality is encountered in this

204 approach if there are errors in classification or if two sequences within the desired threshold belong

205 to different taxonomic groups. It is expected that these errors would increase as the taxonomic level

206 goes from kingdom to genus. To characterize the clustering quality, we classified each sequence

207 at each taxonomic level and calculated the MCC values using OptiClust, average neighbor, and

208 DGC with VSEARCH when splitting at each taxonomic level (Figure 3). For each method, the MCC

209 values decreased as the taxonomic resolution increased; however, the decrease in MCC was not

210 as large as the difference between clustering methods. As the resolution of the taxonomic levels

211 increased, the clustering quality remained high, relative to clusters formed from the entire dataset

212 (i.e. kingdom-level). The MCC values when splitting the datasets at the class and genus levels

213 were within 98.0 and 93.0%, respectively, of the MCC values obtained from the entire dataset.

214 These decreases in MCC value resulted in the formation of as many as 4.7 and 22.5% more OTUs,

215 respectively, than were observed from the entire dataset. These errors were due to the generation

216 of additional false negatives due to splitting similar sequences into different taxonomic groups. For

217 the datasets included in the current analysis, the use of the cluster splitting heuristic was probably

218 not worth the loss in clustering quality. However, as datasets become larger, it may be necessary to

219 use the heuristic to clustering the data into OTUs.

Discussion

221 Myriad methods have been proposed for assigning 16S rRNA gene sequences to OTUs. Each claim

222 improved performance based on speed, memory usage, representation of taxonomic information,

223 and number of OTUs. Each of these metrics is subjective and do not actually indicate the quality

224 of the clustering. This led us to propose using the MCC as a metric for assessing the quality of

225 clustering, post hoc. Here, we described a new clustering method that seeks to optimize clustering

226 based on an objective criterion that measures clustering quality in real time. In the OptiClust

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227 algorithm, clustering is driven by optimizing a metric that assesses whether any two sequences

228 should be grouped into the same OTU. The result is clusters that are significantly more robust and

229 is efficient in the time and memory required to cluster the sequences into OTUs. This makes it

230 more tractable to analyze large datasets without sacrificing clustering quality as was previously

231 necessary using heuristic methods.

232 The cluster optimization procedure is dependent on the metric that is chosen for optimization. We

233 employed the MCC because it includes the four values from a confusion matrix. Other algorithms

234 such as the furthest neighbor and nearest neighbor algorithms minimize the number of FP and

235 FN, respectively; however, these suffer because the number of FN and FP are not controlled,

236 respectively (13, 16). Alternatively, one could optimize based on the sensitivity, specificity, or

237 accuracy, which are each based on two values from the confusion matrix or they could optimize

238 based on the F1-score, which is based on three values from the confusion matrix. Because these

239 metrics do not balance all four parameters equally, it is likely that one parameter will dominate in the

240 optimization procedure. For example, optimizing for sensitivity could lead to a large number of FPs.

241 More FPs increases the number of OTUs while more FNs collapses OTUs together. It is difficult to

242 know which is worse since community richness and diversity are linked to the number of OTUs. In

243 addition, increasing the number of FNs would overstate the differences between communities while

244 increasing the number of FPs would overstate their similarity. Therefore, it is important to jointly

245 minimize the number of FPs and FNs. With this in mind, we decided to optimize utilizing the MCC.

246 It is possible that other metrics that balance the four parameters could be developed and employed

247 for optimization of the clustering.

248 The OptiClust algorithm is relatively simple. For each sequence it effectively asks whether the MCC

249 value will increase if the sequence is moved to a different OTU including creating a new OTU. If the

250 value does not change, it remains in the current OTU. The algorithm repeats until the MCC value

251 stabilizes. Assuming that the algorithm is seeded with each sequence in a separate OTU, it does

252 not appear that the algorithm converges to a local optimum. Furthermore, execution of the algorithm

253 with different random number generator seeds produces OTU assignments of consistently high

254 quality. Future improvements to the implementation of the algorithm could provide optimization to

255 further improve its speed and susceptibility to find a local optimum. Users are encouraged to repeat

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256 the OTU assignment several times to confirm that they have found the best OTU assignments.

257 Our previous MCC-based analysis of clustering algorithms indicated that the average neighbor

258 algorithm consistently produced the best OTU assignments with the DGC-based method using

259 USEARCH also producing robust OTU assignments. The challenge in using the average neighbor

260 algorithm is that it requires a large amount of RAM and is computationally demanding. This led to

261 the development of a splitting approach that divides the clustering across distinct taxonomic groups

262 (13). The improved performance provided by the OptiClust algorithm likely makes such splitting

263 unnecessary for most current datasets. We have demonstrated that although the OTU assignments

264 made at the genus level are still better than that of other methods, the quality is not as good as that

265 found without splitting. The loss of quality is likely due to misclassification because of limitations

266 in the clustering algorithms and reference databases. The practical significance of such small

267 differences in clustering quality remain to be determined; however, based on the current analysis, it

268 does appear that the number of OTUs is artificially inflated. Regardless, the best clustering quality

269 should be pursued given the available computer resources.

270 The time and memory required to execute the OptiClust algorithm scaled proportionally to the

271 number of unique sequences raised to the second power. The power for the time requirement is

272 affected by the similarity of the sequences in the dataset with datasets containing more similar

273 sequences having a higher power. Also, the number of unique sequences is the basis for both the

274 amount of time and memory required to complete the algorithm. Both the similarity of sequences

275 and number of unique sequences can be driven by the sequencing error since any errors will

276 increase the number of unique sequences and these sequences will be closely related to the

277 perfect sequence. This underscores the importance of reducing the noise in the sequence data (7).

278 If sequencing errors are not remediated and are relatively randomly distributed, then it is likely that

279 the algorithm will require an unnecessary amount of time and RAM to complete.

280 The rapid expansion in sequencing capacity has demanded that the algorithms used to assign

281 16S rRNA gene sequences to OTUs be efficient while maintaining robust assignments. Although

282 database-based approaches have been proposed to facilitate this analysis, they are limited by

283 their limited coverage of bacterial taxonomy and by the inconsistent process used to name taxa.

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284 The ability to assign sequences to OTUs using an algorithm that optimizes clustering by directly

285 measuring quality will significantly enhance downstream analysis. The development of the OptiClust

286 algorithm represents a significant advance that is likely to have numerous other applications.

Materials and Methods

288 ***Sequence data and processing steps.*** To evaluate the OptiClust and the other algorithms we

289 created two synthetic sequence collections and four sequence collections generated from previously

290 published studies. The V4 region of the 16S rRNA gene was used from all datasets because it

291 is a popular region that can be fully sequenced with two-fold coverage using the commonly used

292 MiSeq sequencer from Illumina (7). The method for generating the simulated datasets followed

293 the approach used by Kopylova et al. (34) and Schloss (36). Briefly, we randomly selected

294 10,000 uniques V4 fragments from 16S rRNA gene sequences that were unique from the SILVA

295 non-redundant database (38). A community with an even relative abundance profile was generated

296 by specifying that each sequence had a frequency of 100 reads. A community with a staggered

297 relative abundance profile was generated by specifying that the abundance of each sequence was

298 a randomly drawn integer sampled from a uniform distribution between 1 and 200. Sequence

299 collections collected from human feces (39), murine feces (40), soil (41), and seawater (42) were

300 used to characterize the algorithms' performance with natural communities. These sequence

301 collections were all generated using paired 150 or 250 nt reads of the V4 region. We re-processed

302 all of the reads using a common analysis pipeline that included quality score-based error correction

303 (7), alignment against a SILVA reference database (38, 43), screening for chimeras using UCHIME

304 (9), and classification using a naive Bayesian classifier with the RDP training set requiring an 80%

305 confidence score (10).

306 ***Implementation of clustering algorithms.*** In addition to the OptiClust algorithm we evaluated

307 ten different *de novo* clustering algorithms. These included three hierarchical algorithms, average

308 neighbor, nearest neighbor, and furthest neighbor, which are implemented in mothur (v.1.39.0)

309 (11). Seven heuristic methods were also used including abundance-based greedy clustering

310 (AGC) and (distance-based greedy clustering) DGC as implemented in USEARCH (v.6.1) (17) and

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311 VSEARCH (v.2.3.3) ((18)], OTUCLUST (v.0.1) (19), SumaClust (v.1.0.20), and Swarm (v.2.1.9)

312 (20). With the exception of Swarm each of these methods uses distance-based thresholds to report

313 OTU assignments. We also evalauted the ability of OptiClust to optimize to metrics other than

314 MCC. These included accuracy, F1-score, negative predictive value, positive predictive value, false

315 discovery rate, senitivity, specificity, the sum of TPs and TNs, the sum of FPs and FNs, and the

316 number of FNs, FPs, TNs, and TPs (Figure S1; Table S1).

317 **Benchmarking.** We evaluated the quality of the sequence clustering, reproducibility of the

318 clustering, the speed of clustering, and the amount of memory required to complete the clustering.

319 To assess the quality of the clusters generated by each method, we counted the cells within a

320 confusion matrix that indicated how well the clusterings represented the distances between the pair

321 of sequences (13). Pairs of sequences that were in the same OTU and had a distance less than

322 3% were true positives (TPs), those that were in different OTUs and had a distance greater than

323 3% were true negatives (TNs), those that were in the same OTU and had a distance greater than

324 3% were false positives (FPs), and those that were in different OTUs and had a distance less than

325 3% were false negatives (FNs). To synthesize the matrix into a single metric we used the Matthews

326 correlation coefficient using the `sens.spec` command in mothur using the following equations.

$$MCC = \frac{TP \times TN - FP \times FN}{\sqrt{(TP + FP)(TP + FN)(TN + FP)(TN + FN)}}$$

327 To assess the reproducibility of the algorithms we randomized the starting order of each sequence

328 collection ten times and ran each algorithm on each randomized collection. We then measured the

329 MCC for each randomization and quantified their percent coefficient of variation (% CV; 100 times

330 the ratio of the standard deviation to the mean).

331 To assess how the the memory and time requirements scaled with the number of sequences

332 included in each sequence collection, we randomly subsampled 20, 40, 60, or 80% of the unique

333 sequences in each collection. We obtained 10 subsamples at each depth for each dataset and ran

334 each collection (N= 50 = 5 sequencing depths x 10 replicates) through each of the algorithms. We

335 used the `timeout` script to quantify the maximum RAM used and the amount of time required to

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336 process each sequence collection (https://github.com/pshved/timeout). We limited each algorithm

337 to 45 GB of RAM and 50 hours using a single processor.

338 **_Data and code availability._** The workflow utilized commands in GNU make (v.3.81), GNU bash

339 (v.4.1.2), mothur (v.1.39.0) (11), and R (v.3.3.2) (44). Within R we utilized the wesanderson (v.0.3.2)

340 (45), dplyr (v.0.5.0) (46), tidyr (v.0.6.0) (47), cowplot (v.0.6.3) (48), and ggplot2 (v.2.2.0.9000) (49)

341 packages. A reproducible version of this manuscript and analysis is available at https://github.com/

342 SchlossLab/Westcott_OptiClust_mSphere_2017.

Acknowledgements

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Table 1. Description of datasets used to evaluate the OptiClust algorithm and compare its performance to other algorithms. Each dataset contains sequences from the V4 region of the 16S rRNA gene. The number of distances for each dataset are those that were less than or equal to 0.03. The number of OTUs were determined using the OptiClust algorithm. The even and staggered datasets were generated by extracting the V4 region from full length reference sequences and the datasets from the natural communities were generated by sequencing the V4 region using a Illumina MiSeq with either paired 150 or 250 nt reads.

| Dataset (Ref.)	Read Length	Samples	Total Seqs.	Unique Seqs.	Distances	OTUs
Soil (41)	150	18	948,243	143,677	11,775,167	40,216
Marine (42)	250	7	1,384,988	75,923	12,908,857	25,787
Mice (40)	250	360	2,825,495	32,447	6,988,306	2,658
Human (39)	250	489	20,951,841	121,281	38,544,315	11,648
Even (34, 36)	NA	NA	1,155,800	11,558	29,694	7,651
Staggered (34, 36)	NA	NA	1,156,550	11,558	29,694	7,653

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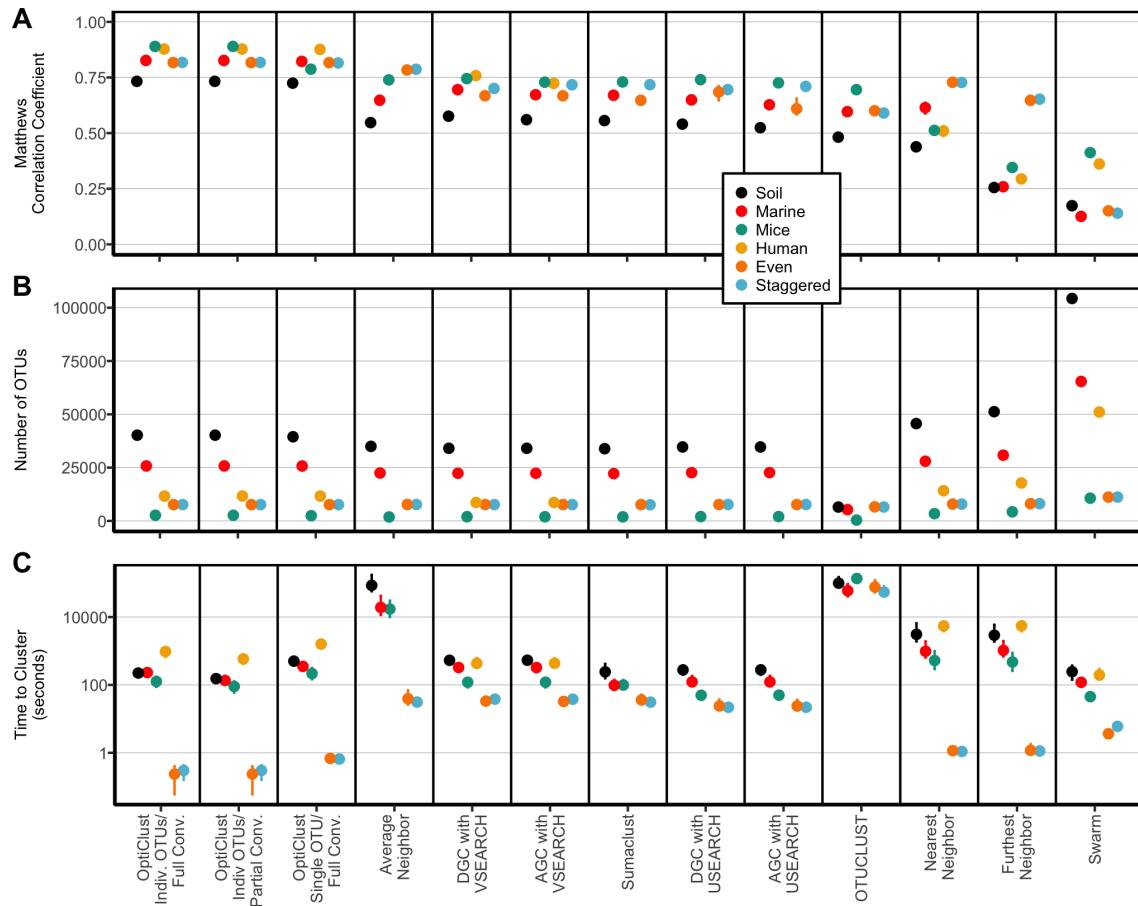
Figure 1. Comparison of de novo clustering algorithms. Plot of MCC (A), number of OTUs (B), and execution times (C) for the comparison of *de novo* clustering algorithms when applied to four natural and two synthetic datasets. The first three columns of each figure contain the results of clustering the datasets (i) seeding the algorithm with one sequence per OTU and allowing the algorithm to proceed until the MCC value no longer changed; (ii) seeding the algorithm with one sequence per OTU and allowing the algorithm to proceed until the MCC changed by less than 0.0001; (iii) seeding the algorithm with all of the sequences in one OTU and allowing the algorithm to proceed until the MCC value no longer changed. The human dataset could not be clustered by the average neighbor, Sumaclust, USEARCH, or OTUCLUST with less than 45 GB of RAM or 50 hours of execution time. The median of 10 re-orderings of the data is presented for each method and dataset. The range of observed values is indicated by the error bars, which are typically smaller than the plotting symbol.

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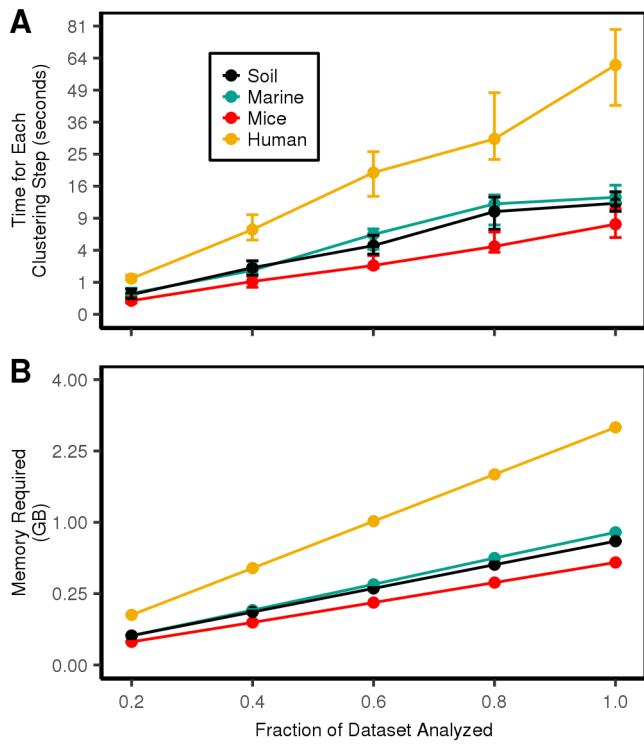
Figure 2. OptiClust performance The average execution time (A) and memory usage (B) required to cluster the four natural datasets. The confidence intervals indicate the range between the minimum and maximum values. The y-axis is scaled by the square root to demonstrate the relationship between the time and memory requirements relative to the number of unique sequences squared.
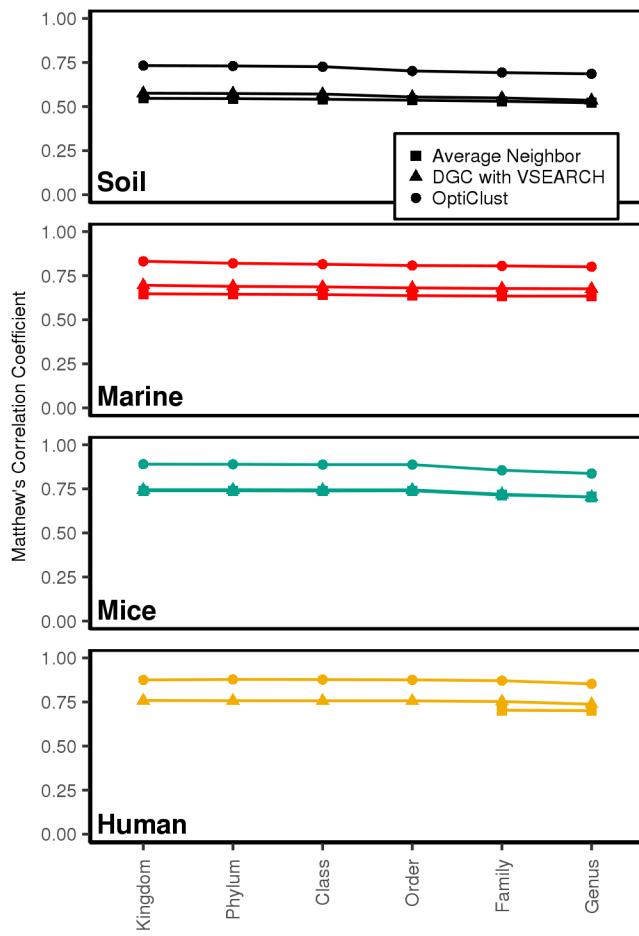
Figure 3. Effects of taxonomically splitting the datasets on clustering quality. The datasets were split at each taxonomic level based on their classification using a naive Bayesian classifier and clustered using average neighbor, VSEARCH-based DGC, and OptiClust.

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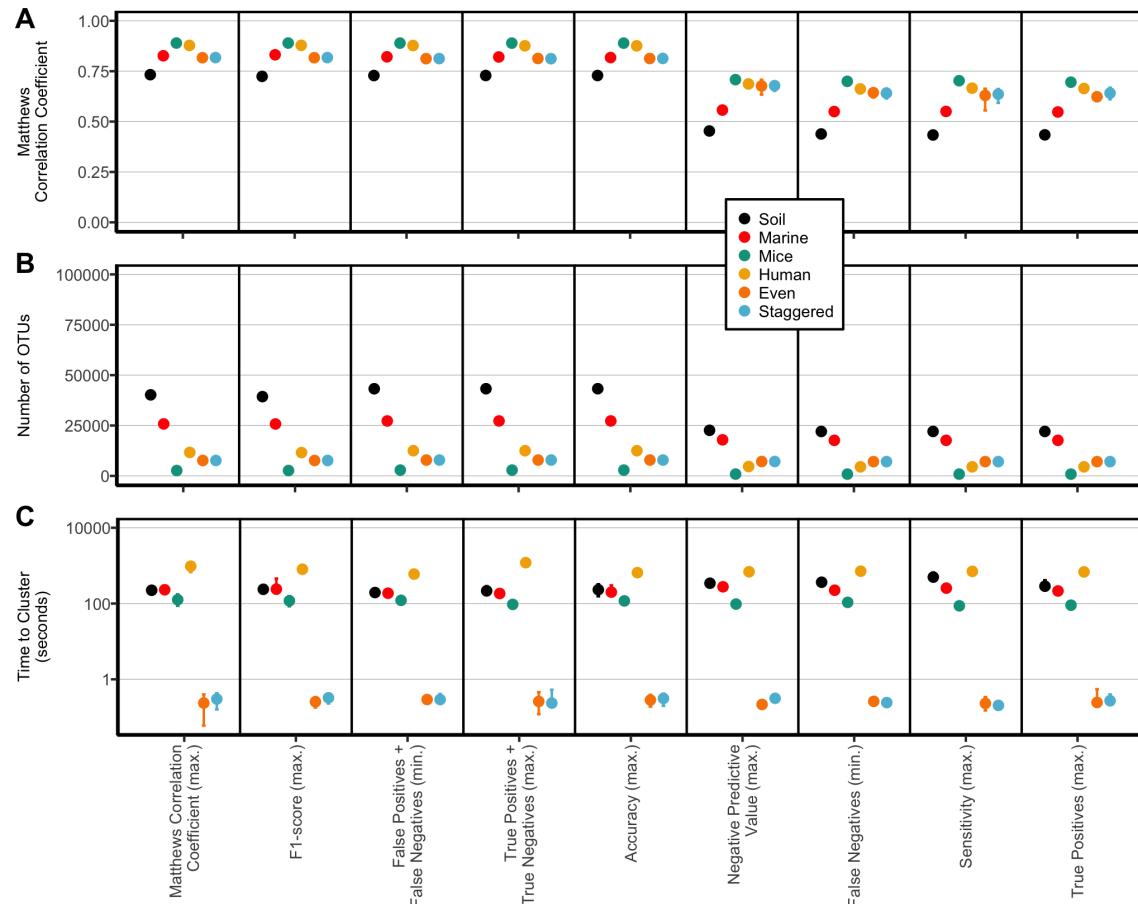
Figure S1. The OptiClust algorithm is able to effectively cluster sequences into OTUs by minimizing or maximizing numerous metrics. Plot of MCC (A), number of OTUs (B), and execution times (C) for the comparison of output from the OptiClust algorithm when to minimizing or maximizing a variety of parameters when applied to four natural and two synthetic datasets. Within mothur, OTU assignments can also be made using other metrics including minimizing false positives and maximizing the specificity, positive predictive value, and true negatives; however, these all resulted in sequences being assigned to separate OTUs, which resulted in no false positives and the maximum number of true negatives. The error bars indicate the range of values observed for 10 replicates.

387
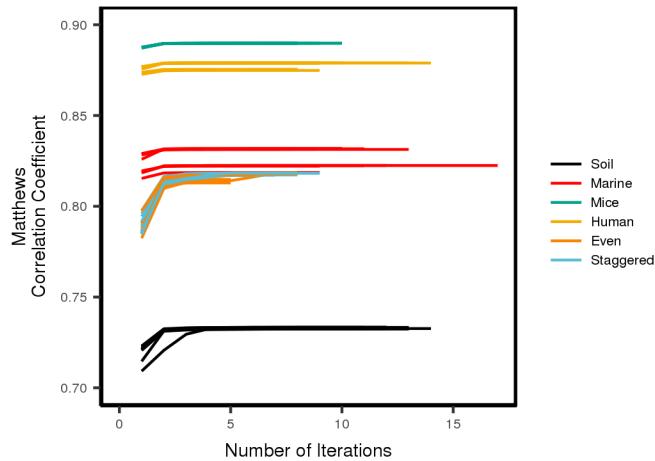
Figure S2. The OptiClust algorithm rapidly converges to optimize the Matthews correlation coefficient. The six datasets were clustered into OTUs using the OptiClust algorithm seeking to maximize the Matthews correlation coefficient. This was repeated 10 times for each dataset.

391 **Supplemental text.** Worked example of how OptiClust algorithm clusters sequences into OTUs.

392 **Table S1. Summary of the average number of true positives, true negatives, false positives,**

393 **false negatives and the resulting Matthews correlation coefficient for each of the clustering**

394 **methods that were analyzed in this study for each of the six datasets.** Blank values indicate

395 that those conditions could not be completed in 50 hours with 45 GB of RAM.

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Table S1. Summary of the average number of true positives, true negatives, false positives, false negatives and the resulting Matthews correlation coefficient for each of the clustering methods that were analyzed in this study for each of the six datasets. Blank values indicate that those conditions could not be completed in 50 hours with 45 GB of RAM.

Dataset	Method	True Positives	True Negatives	False Positives	False Negatives	MCC
Soil	MCC (separate OTUs)	5136712	10312528053	1247993	2555568	0.7328
Soil	TP + TN	4770846	10312976362	799684	2921434	0.7287
Soil	Accuracy	4771037	10312974895	801151	2921243	0.7286
Soil	FPs + FNs	4768761	10312976641	799405	2923519	0.7285
Soil	MCC (single OTU)	5399187	10311962308	1813738	2293093	0.7247
Soil	F1-score	5453578	10311862032	1914014	2238702	0.7244
Soil	VSEARCH (w/AGC)	5953502	10305863000	7913046	1738778	0.5760
Soil	VSEARCH (w/AGC)	6684050	10301990342	11785704	1008230	0.5603
Soil	Sumaclust	6689597	10301700186	12075860	1002683	0.5563
Soil	Average Neighbor	6746206	10300797169	12978877	946074	0.5472
Soil	USEARCH (w/DGC)	4851924	10308165644	5610402	2840356	0.5404
Soil	USEARCH (w/AGC)	5592316	10304608407	9167639	2099964	0.5244
Soil	OTUCLUST	6714016	6909706980	18444775	978264	0.4819
Soil	Negative Predictive Value	7382082	10286785610	26990436	310198	0.4534
Soil	FN	7438201	10283907614	29868432	254079	0.4386
Soil	Nearest Neighbor	1571726	10313677482	98564	6120554	0.4383
Soil	Sensitivity	7440261	10283040048	30735998	252019	0.4335
Soil	Furthest Neighbor	501819	10313776046	0	7190461	0.2553
Soil	Swarm	237705	10313770690	5356	7454575	0.1738
Marine	F1-score	7914666	2870997648	1894417	1306272	0.8317
Marine	MCC (separate OTUs)	7670655	2871231788	1660277	1550283	0.8266
Marine	MCC (single OTU)	7428998	2871482154	1409911	1791940	0.8223
Marine	FPs + FNs	7264205	2871683595	1208470	1956733	0.8214
Marine	TP + TN	7211154	2871739258	1152807	2009784	0.8206
Marine	Accuracy	7119498	2871802386	1089679	2101440	0.8178
Marine	VSEARCH (w/AGC)	8566881	2865055591	7836474	654057	0.6954
Marine	VSEARCH (w/AGC)	8867443	2862976753	9915312	353495	0.6725
Marine	Sumaclust	8867317	2862856105	10035960	353621	0.6703
Marine	USEARCH (w/DGC)	6929948	2867522405	5369660	2290990	0.6494
Marine	Average Neighbor	8914621	2861348612	11543453	306317	0.6476
Marine	USEARCH (w/AGC)	7352302	2865419824	7472241	1868636	0.6274
Marine	Nearest Neighbor	3742329	2872622032	270033	5478609	0.6144
Marine	OTUCLUST	8945456	1834755673	15224413	275482	0.5965
Marine	Negative Predictive Value	9131188	2853099098	19792967	89750	0.5572
Marine	Sensitivity	9136046	2852367594	20524471	84892	0.5504
Marine	FN	9143826	2852250720	20641345	77112	0.5498
Marine	Furthest Neighbor	620264	2872892065	0	8600674	0.2589
Marine	Swarm	149810	2872888019	4046	9071128	0.1256
Mice	MCC (separate OTUs)	4829770	520373752	491600	692559	0.8898
Mice	F1-score	4850792	520347542	517810	671537	0.8897
Mice	FPs + FNs	4771312	520439019	426333	751016	0.8895
Mice	TP + TN	4770937	520439150	426202	751392	0.8894
Mice	Accuracy	4769575	520440407	424945	752754	0.8894
Mice	MCC (single OTU)	4879087	518832093	2033259	643242	0.7873
Mice	VSEARCH (w/AGC)	5100191	517549332	3316020	422138	0.7450
Mice	USEARCH (w/DGC)	4905962	517883072	2982280	616367	0.7402
Mice	Average Neighbor	5326219	516873116	3992236	196110	0.7393
Mice	Sumaclust	5317976	516662498	4202854	204353	0.7301
Mice	VSEARCH (w/AGC)	5322737	516622070	4243282	199592	0.7290
Mice	USEARCH (w/AGC)	5243162	516749770	4115582	279167	0.7260
Mice	Negative Predictive Value	5426962	515767988	5097364	95366	0.7082
Mice	Sensitivity	5439118	515571271	5294081	83211	0.7028
Mice	FN	5451490	515436798	5428554	70839	0.6996
Mice	OTUCLUST	5297141	469202414	5087312	225188	0.6953
Mice	Nearest Neighbor	1482308	520843906	21446	4040021	0.5122
Mice	Swarm	1237099	520494387	370965	4285230	0.4123
Mice	Furthest Neighbor	665512	520865352	0	4856816	0.3455
Human	F1-score	26073348	7321213423	2922678	4270391	0.8785
Human	MCC (separate OTUs)	25861724	7321423671	2712430	4482015	0.8778
Human	FPs + FNs	25512998	7321813377	2322724	4830740	0.8774
Human	MCC (single OTU)	25680854	7321538561	2597540	4662885	0.8762
Human	TP + TN	25399667	7321870325	2265776	4944072	0.8762
Human	Accuracy	25352243	7321910658	2225443	4991496	0.8759
Human	VSEARCH (w/AGC)	26845545	7309824393	14311708	3498194	0.7585
Human	VSEARCH (w/AGC)	28561130	7301551637	22584464	1782609	0.7237
Human	Negative Predictive Value	29719012	7292417394	31718707	624727	0.6867
Human	Sensitivity	29818923	7288099634	36036467	524816	0.6657
Human	FN	29888157	7287209148	36926953	455582	0.6623
Human	Nearest Neighbor	8062473	7323981460	154641	22281266	0.5097
Human	Swarm	4358052	7323725163	410938	25985688	0.3615
Human	Furthest Neighbor	2637845	7324136101	0	27705894	0.2942
Human	Average Neighbor					
Human	OTUCLUST					
Human	Sumaclust					
Human	USEARCH (w/AGC)					
Human	USEARCH (w/DGC)					
Even	MCC (separate OTUs)	17174	66762945	2465	5319	0.8171
Even	F1-score	17586	66762397	3013	4907	0.8169
Even	MCC (single OTU)	17174	66762930	2480	5319	0.8168
Even	TP + TN	16378	66763755	1655	6115	0.8132
Even	Accuracy	16349	66763784	1626	6144	0.8130
Even	FPs + FNs	16312	66763795	1615	6181	0.8123
Even	Average Neighbor	15607	66763393	2017	6886	0.7838
Even	Nearest Neighbor	12448	66764886	524	10046	0.7287
Even	USEARCH (w/DGC)	12950	66762468	2942	9544	0.6847
Even	Negative Predictive Value	20746	66744250	21160	1747	0.6768
Even	VSEARCH (w/AGC)	12340	66762579	2831	10153	0.6679
Even	VSEARCH (w/AGC)	12340	66762579	2831	10153	0.6679
Even	Furthest Neighbor	9434	66765410	0	13059	0.6475
Even	Sumaclust	12559	66761243	4167	9934	0.6474
Even	FN	21047	66738879	26531	1446	0.6436
Even	Sensitivity	21106	66735974	29436	1387	0.6299
Even	USEARCH (w/AGC)	11260	66761521	3889	11233	0.6096
Even	OTUCLUST	16844	66725346	18107	5649	0.6007
Even	Swarm	520	66765401	9	21973	0.1507
Staggered	MCC (separate OTUs)	17069	66763106	2304	5424	0.8176
Staggered	F1-score	17584	66762432	2978	4909	0.8176
Staggered	MCC (single OTU)	17200	66762827	2583	5293	0.8154
Staggered	Accuracy	16337	66763813	1597	6156	0.8134
Staggered	FPs + FNs	16313	66763811	1599	6180	0.8127
Staggered	TP + TN	16319	66763779	1631	6174	0.8121
Staggered	Average Neighbor	16142	66762854	2556	6351	0.7871
Staggered	Nearest Neighbor	12413	66764886	524	10080	0.7276
Staggered	Sumaclust	15377	66760390	5020	7116	0.7178
Staggered	VSEARCH (w/AGC)	15095	66760823	4587	7398	0.7173
Staggered	USEARCH (w/AGC)	14630	66761174	4236	7863	0.7101
Staggered	VSEARCH (w/AGC)	13567	66762304	3106	8926	0.7005
Staggered	USEARCH (w/DGC)	13244	66762527	2873	9250	0.6955
Staggered	Negative Predictive Value	20724	66744588	20822	1769	0.6783
Staggered	Furthest Neighbor	9575	66765410	0	12918	0.6524
Staggered	FN	21018	66738538	26872	1475	0.6413
Staggered	Sensitivity	20981	66737899	27511	1512	0.6361
Staggered	OTUCLUST	15625	66371336	15527	6868	0.5903
Staggered	Swarm	443	66765408	2	22050	0.1400

Supplemental Text

To provide a detailed demonstration of how the OptiClust algorithm works consider a relatively simple example where there are 50 sequences. After aligning the sequences and calculating the pairwise distances between the sequences there are 15 pairs of sequences with a distance below the desired threshold of 0.03:

Seq. 1	Seq. 2	Distance
D	B	0.024
F	E	0.028
G	C	0.028
H	A	0.027
I	B	0.016
I	D	0.024
J	A	0.028
J	H	0.024
N	M	0.024
O	L	0.024
P	K	0.016
P	L	0.027
P	O	0.027
Q	E	0.024
Q	F	0.028

The other 1210 distances were larger than 0.03 and are not needed for the analysis because they are taken to be true negatives. For example, the distance between sequences A and B is larger than 0.03. So, if they are in different OTUs then that would be a true negative (TN) and if they are in the same OTU then that would be a false positive (FP). Alternatively, because sequences B and D are closer to each other than 0.03 (i.e. 0.024) if they are in separate OTUs then that would be a false negative (FN) and if they are in the same OTU then that would be a true positive (TP). It is important to note that the algorithm assumes that there are no duplicate sequences and the actual abundance is saved elsewhere to be substituted later when counting the frequency distribution of each OTU across the samples included in the analysis.

The algorithm starts by seeding sequences either into individual OTUs or into a single OTU. As demonstrated in Figure 1, seeding the sequences into randomly ordered individual OTUs generates better results and is faster than starting with a single OTU. Among the 15 pairwise distances that are smaller than 0.03, there are 17 sequences that are labeled A through Q. A separate pool is created for the 33 other sequences that are not within 0.03 of any other sequence and are thus to be placed into 33 separate OTUs. In the diagrams below, this pool is designated as "…". Having seeded the initial OTUs there are 0 TPs, 1210 TNs, 0 FPs, and 15 FNs. Initially the number of FNs corresponds to the number of distances less than 0.03, the number of TNs is the number of total distances (i.e. 1225) minus the number of distances less than 0.03. The number of TPs, TNs, FPs, and FNs should sum to the total number of distances. The resulting Matthew's Correlation Coefficient (MCC) is 0.00. The algorithm next goes through each sequence sequentially to determine whether the MCC

1

value would be increased by removing it from its current OTU to join other sequences in a new OTU or to create its own OTU.

The demonstration of the algorithm starts with sequence A. Notice that it is within 0.03 of sequences H and J. There are three options: sequence A could remain as its own OTU, it could join with sequence H, or it could join with sequence J.
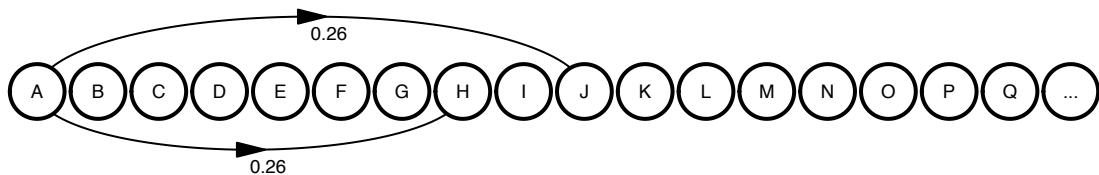

If it stays as its own OTU, the MCC value would remain 0.00. If it joined with sequences H or J the number of TPs would increase by 1 and the number of FNs would decrease by 1. In either case the MCC would be 0.26. In this case, joining H or J would result in an improved MCC and so the algorithm randomly selects which sequences to join. For this demonstration, it will form a new OTU with sequence H. This results in 1 TPs, 1210 TNs, 0 FPs, 14 FNs, and an MCC of 0.26.

Sequence B is processed by the same process as sequence A. Sequence B is within 0.03 of sequences D and I. Again, there are three options: sequence B could remain as its own OTU, it could join with sequence D, or it could join with sequence I.
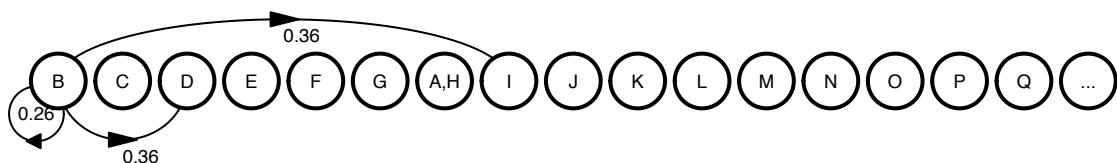
If it remains as its own OTU, the MCC value would remain 0.26. If it joined with sequences D or I the number of TPs would increase by 1 and the number of FNs would decrease by 1. In either case the MCC would be 0.36. Joining D or I would result in an improved MCC and so the algorithm randomly selects which sequences to join. For this demonstration it will form a new OTU with sequence D. This results in 2 TPs, 1210 TNs, 0 FPs, 13 FNs, and an MCC of 0.36.

Sequence C is within 0.03 of sequence G creating two options: remain as its own OTU or join with sequence G.

2
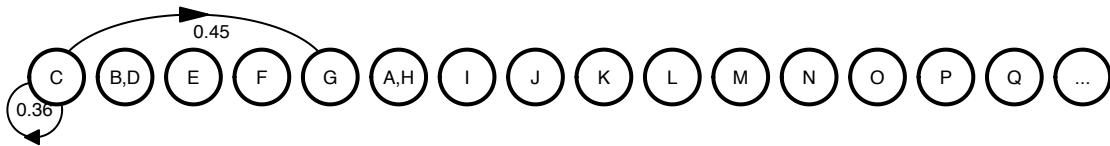
If it remains in its own OTU, the MCC value would remain 0.36. If it joined with sequence G the number of TPs would increase by 1 and the number of FNs would decrease by 1 resulting in an MCC value of 0.45. Because of the improved MCC value, sequence C joins with sequence G to form a new OTU. This results in 3 TPs, 1210 TNs, 0 FPs, 12 FNs, and an MCC of 0.45.

For sequence D the algorithm gets more complicated since sequence D is already in an OTU with sequence B. As seen when considering sequence B, sequence D is within 0.03 of sequence B and it is also similar to sequence I. Now there are three options: sequence D could remain with sequence B in an OTU, it could leave that OTU and join with sequence I, or it could form a new OTU where it is the sole member.
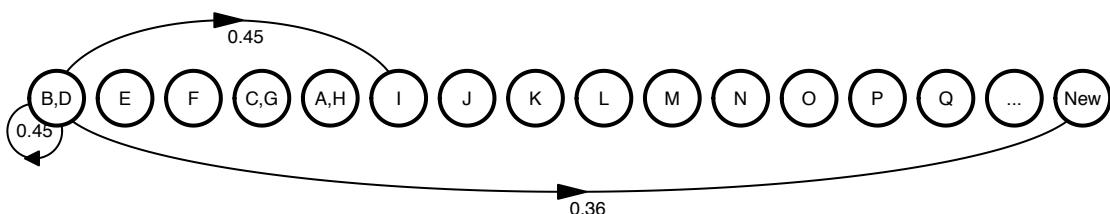
If sequence D remains with B, the MCC value would remain 0.45. If it joined with sequence I the number of TPs and FNs would stay constant resulting in an MCC value of 0.51. If it formed a new OTU by itself, the number of TPs would decrease by one and the number of FNs would increase by 1 resulting in an MCC value of 0.36. Because the MCC values for staying in the OTU with B or leaving the OTU to join the OTU with I are the same, the algorithm would again randomly chose between the two options. For demonstration purposes, sequence D will remain in its OTU with sequence B. This results in no changes in the four parameters or the MCC value.

For sequence E, the same type of options are available as when sequences A and B were processed. Sequence E could remain on its own or it could join with sequences F or Q.

3
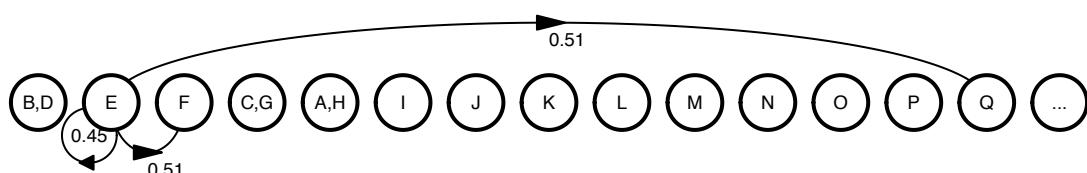
Similar to the earlier cases, the MCC value for joining another sequence is larger than staying on its own. Because the MCC values for joining F or Q are the same, the algorithm randomly selects which sequences to join. For this demonstration sequence E will form a new OTU with sequence F. This results in 4 TPs, 1210 TNs, 0 FPs, 11 FNs, and an MCC of 0.51.

For sequence F, the steps taken by the algorithm are the same as earlier for sequence D. Here, sequence F could remain in an OTU with sequence E, it could leave and form an OTU with sequence Q, or it could form a new OTU on its own.
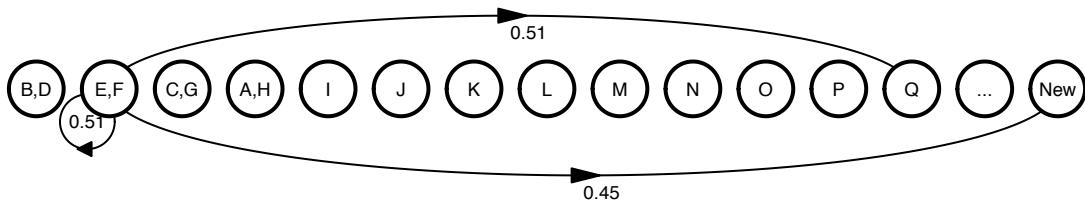
Again, the MCC value for sequence F remaining with sequence E is the same as for leaving to form an OTU with sequence Q. Both options are superior to leaving to form a new OTU on its own. The algorithm randomly chooses between the two options. For demonstration purposes, sequence F will remain in its OTU with sequence E. This results in no changes in the four parameters or the MCC value.

The decisions for sequence G are similar to sequence F, with the exception that the only choices are to stay in an OTU with another sequence (C) or to form a new OTU on its own.
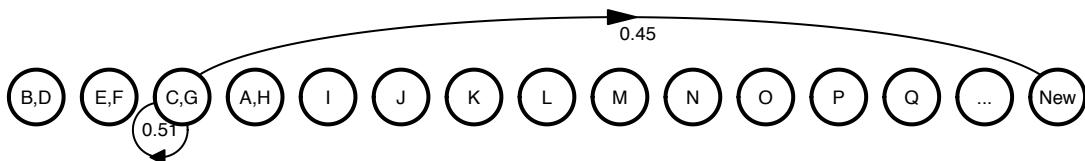
Leaving to form a new OTU results in a lower MCC value and so the algorithm leaves sequence G with sequence C. This results in no changes in the four parameters or the MCC value.

For sequence H the steps taken are the same as seen earlier for sequence B.
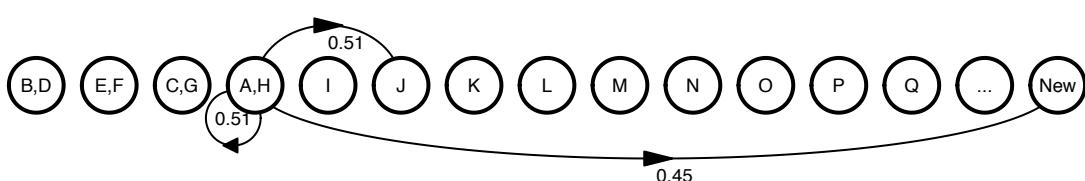
For demonstration purposes sequence H will remain in its OTU with sequence A.

Moving to sequence I, the process is similar to what was done earlier with sequences A and B. The only difference is that because sequence I is similar to both sequences B and D the increase in TP and decrease in FN will be double.
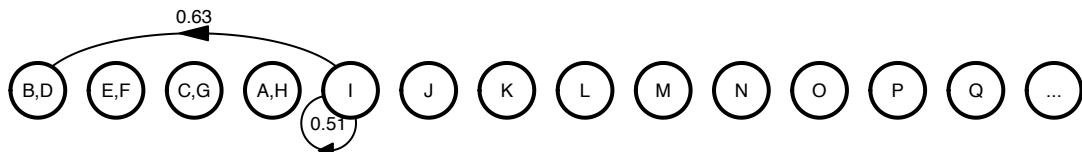

If sequence I remains in an OTU by itself, the MCC value will be 0.51. If it joined the OTU with sequences B and D, then the number of TPs would increase by 2 and the number of FNs would decrease by 2 resulting in an improved MCC value of 0.63. This is the choice that is taken resulting in 6 TPs, 1210 TNs, 0 FPs, and 9 FNs.

Processing sequence J is the same as sequence I since sequence J is close to both A and H.
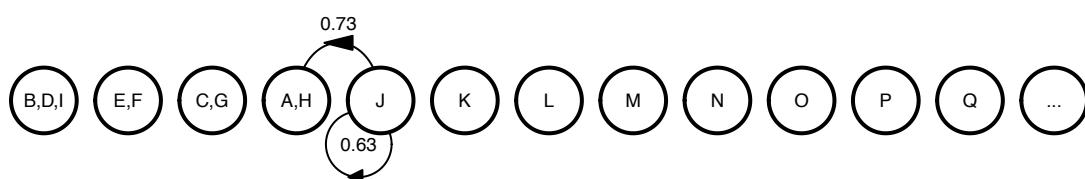
Again, by joining the OTU containing sequences A and H the number of TPs increases by 2 and the number of FNs decreases by 2 resulting in 8 TPs, 1210 TNs, 0 FPs, 7 FNs, and an improved MCC value of 0.73.

Processing sequence K is the same as for sequence A.
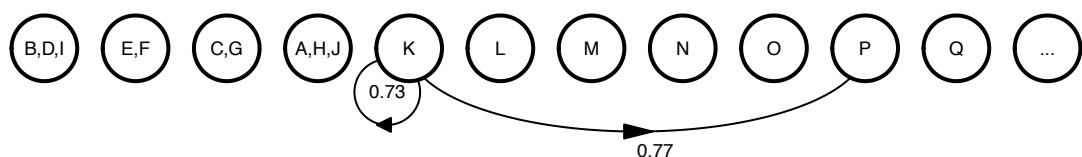
Merging sequences K and P into the same OTU results in 9 TPs, 1210 TNs, 0 FPs, 6 FNs, and an improved MCC value of 0.77.

Processing sequence L presents a more complicated set of decisions. Again, there are three choices. Because sequence L is similar to sequences O and P it could form an OTU with sequence O or with the OTU containing sequences K and P.
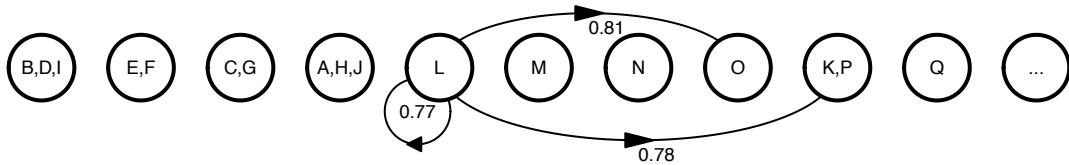

If sequence L remains on its own, the MCC value would remain 0.77. If it joined with sequence O the number of TPs would increase by 1 and the number of FNs would decrease by 1 resulting in an MCC value of 0.81. The subtlety of this step is found in when considering the possibility of sequence L joining an OTU with sequences K and P. It would increase the number of TPs by one and decrease the number of FN by one and by joining with sequence P; however, because O is not close to K the number of TNs would decrease by one and the number of FPs would increase by one. This would result in an MCC value of 0.78. Of the three options forming an OTU with sequences L and O provides the maximal MCC value. This results in 10 TPs, 1210 TNs, 0 FPs, 5 FNs, and an improved MCC value of 0.81.

The steps for processing sequence M is the same as earlier for sequence C.
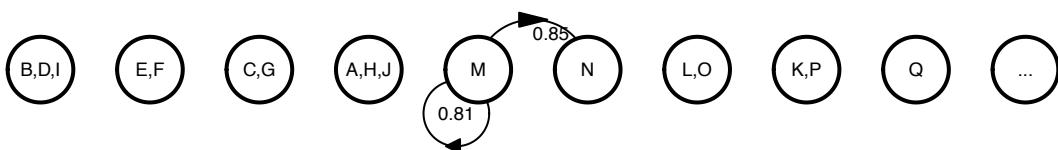

Merging sequences M and N into the same OTU results in 11 TPs, 1210 TNs, 0 FPs, 4 FNs, and an improved MCC value of 0.85.

Moving on to sequence N, the two options are to stay in an OTU with sequence M or to spit off and form a new OTU on its own.


6
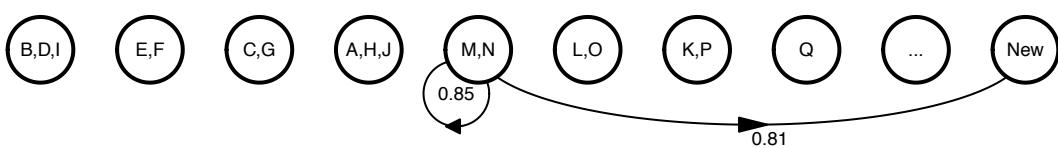
Remaining in an OTU with sequence M provides the larger MCC value and so the OTU memberships do not change.

Processing of sequence O presents three options that have been explored before. Sequence O can stay in its OTU with sequence L, it can join the OTU with sequences K and P, or it can form a new OTU on its own.
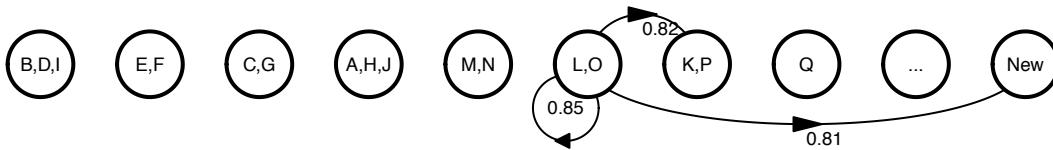

If sequence O remains in the OTU with sequence L, the MCC value would be 0.85. If it leaves that OTU to join sequences K and P in their OTU then the number of TNs would decrease by 1, but the number of FPs would increase by 1 because O is similar to P, but not to K. This would result in an MCC value of 0.82. If sequence O forms a new OTU on its own, then the number of TPs would decrease by one and the number of FNs would increase by one resulting in an MCC value of 0.81. The best option is for sequence O to remain in its OTU with sequence L.

For sequence P the steps taken are similar to those used to evaluate clusters for sequence O; however, the final decision is different. Sequence P can stay with sequence K in their OTU, it can leave to join the OTU with sequences L and O, or it can form a new OTU on its own.
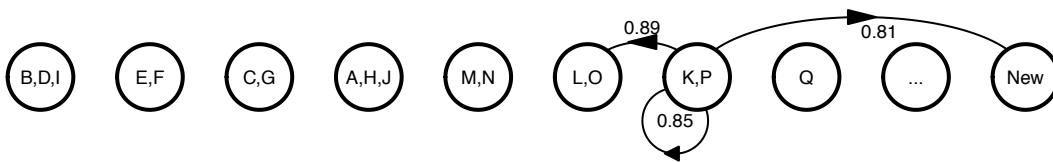

If sequence P remains in the OTU with sequence K, the MCC value would be 0.85. Alternatively, P could leave that OTU to join sequences L and O in their OTU. If P leaves its OTU with K then the number of TPs would decrease by one and the number of FNs would increase by one. By joining with L and O the number of TPs would increase by two and the number of FNs would decrease by two. The net effect would be to increase the number of TPs by 1 and decrease the number of FNs by 1. This would result in an MCC value of 0.89. If sequence P formed a new OTU on its own, then the number of TPs would decrease by one and the number of FNs would increase by one resulting in an MCC value of 0.81. The best option is for sequence P to leave its OTU with sequence K and join the OTU containing sequences L and O. The updated counts are 12 TPs, 1210 TNs, 0 FPs, 3 FNs.

To finish the first round of processing each sequence, sequence Q is processed. Sequence Q is similar to both sequences E and F. Because sequences E and F are in the same OTU, the situation is similar to processing sequence I.
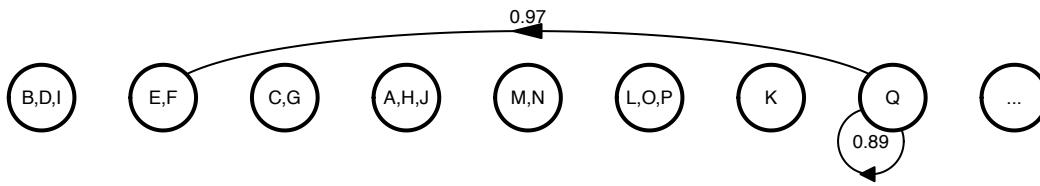
By joining the OTU containing sequences E and F the number of TPs increases by two and the number of FNs decreases by one. The updated counts are 14 TPs, 1210 TNs, 0 FPs, and 1 FNs, which result in an improved MCC value of 0.97.

Having processed each sequence, the first iteration of the algorithm is complete. The MCC value has changed from 0.00 to 0.97. Because the MCC value changed, it is necessary to re-evaluate each sequence again and re-evaluate the final MCC value to determine whether it has changed. In this case, evaluation of sequences A through J result in the same clustering pattern. When the algorithm reaches sequence K it finds that the sequence is similar to sequence P, which is in an OTU with L and O; however sequence K is not similar to L or O.
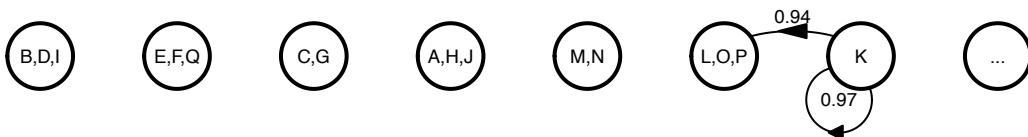
Although, sequence K is similar to sequence P, it is not similar to sequences L or O. Were sequence K to join their OTU, it would increase the number of TPs by one and decrease the number of FNs by one because of its similarity to sequence P, but it would increase the number of FPs by two and decrease the number of TNs by two because K is not similar to L or O. The end result would be a MCC value of 0.94, which is less than the MCC value of keeping sequence K on its own (i.e. 0.97).

Continuing the process for the remaining sequences, none of the sequences will move between OTUs and the MCC value does not change. At this point, the clustering has converged to the optimum MCC value of 0.97. Repeating this process using 100 different seeds for the random number generator required a median of 3 iterations (range from 2 to 4) to converge.

8