Consistent, comprehensive and computationally efficient OTU definitions.

We present a performance-optimized algorithm, subsampled open-reference OTU picking, for assigning marker gene (e.g., 16S rRNA) sequences generated on next-generation sequencing platforms to operational taxonomic units (OTUs) for microbial community analysis. This algorithm provides benefits over de novo OTU picking (clustering can be performed largely in parallel, reducing runtime) and closed-reference OTU picking (all reads are clustered, not only those that match a reference database sequence with high similarity). Because more of our algorithm can be run in parallel relative to "classic" open-reference OTU picking, it makes open-reference OTU picking tractable on massive amplicon sequence data sets (though on smaller data sets, "classic" open-reference OTU clustering is often faster). We illustrate that here by applying it to the first 15,000 samples sequenced for the Earth Microbiome Project (1.3 billion V4 16S rRNA amplicons). To the best of our knowledge, this is the largest OTU picking run ever performed, and we estimate that our new algorithm runs in less than 1/5 the time than would be required of "classic" open reference OTU picking. We show that subsampled open-reference OTU picking yields results that are highly correlated with those generated by "classic" open-reference OTU picking through comparisons on three well-studied datasets. An implementation of this algorithm is provided in the popular QIIME software package, which uses uclust for read clustering. All analyses were performed using QIIME's uclust wrappers, though we provide details (aided by the open-source code in our GitHub repository) that will allow implementation of subsampled open-reference OTU picking independently of QIIME (e.g., in a compiled programming language, where runtimes should be further

reduced). Our analyses should generalize to other implementations of these OTU picking algorithms. Finally, we present a comparison of parameter settings in QIIME's OTU picking workflows and make recommendations on settings for these free parameters to optimize runtime without reducing the quality of the results. These optimized parameters can vastly decrease the runtime of uclust-based OTU picking in QIIME.

Consistent, comprehensive and computationally efficient OTU definitions

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Introduction

Three high-level strategies for defining Operational Taxonomic Unit (OTU) cluster centroids have been widely applied for centroid-based greedy clustering (Li and Godzik 2006; Edgar 2010) of marker gene (e.g., 16S rRNA) sequences generated on next-generation sequencing platforms to facilitate microbial community analysis. These are canonically described as *de novo*, closed-reference, and open-reference OTU picking (Navas-Molina et al. 2013). In each of these approaches, respectively, centroids are defined internally based only on the sequences being clustered, based only on an external, predefined database of cluster centroids, or based on a combination of the two. Each of these methods has benefits and drawbacks.

In *de novo* OTU picking, input sequences are aligned against one another, and sequences that align with greater than a user-specified percent identity are defined as belonging to the same OTU. There are many variations and free parameters in this process, such as how many alignments are performed before a sequence is assigned to an OTU or used to define a new OTU, but the common feature of these methods is that no external reference database is required. This is also the primary advantage of this method: it is not necessary to have accumulated a collection

41   of reference sequences before working with a new marker gene. However, *de novo* OTU picking
42   is difficult to parallelize because all processes must be able to use new OTUs that are defined by
43   other processes. Consequently, this approach cannot scale to modern-sized data sets.

44   In closed-reference OTU picking, input sequences are aligned to pre-defined cluster centroids in
45   a reference database. If the input sequence does not match any reference sequence at a user-
46   defined percent identity threshold, that sequence is excluded. The primary advantage of closed-
47   reference OTU picking is that it is easily parallelizable. Because the cluster centroids are
48   predefined, the input sequence collection can be partitioned into *n* subsets, the assignment
49   process can be split across *n* processors, and the clustering results can be collated when all
50   processes have completed. This dramatically reduces the "wall time" (i.e., the total time to
51   completion as you would see it on a clock on the wall, not in terms of CPU × hours) of this
52   method, and makes closed-reference OTU picking a convenient strategy for extremely large
53   datasets (e.g, as in (Yatsunenko et al. 2012)). Additionally, it has the convenient feature that,
54   because OTUs are defined by a pre-existing reference, there are typically high-quality taxonomic
55   assignments for each OTU, and a high-quality phylogenetic tree, often based on full-length
56   sequences rather than fragments, exists and describes the relationships among those OTUs.
57   Furthermore, because input sequences are not compared directly to one another, but rather to an
58   external reference, the input sequences need not overlap. This is essential, for example, if
59   performing a meta-analysis including sequences derived from different amplification products of
60   the same marker gene, such as the V2 and V4 regions of the 16S rRNA (e.g., as in the meta-
61   analysis performed in (Caporaso et al. 2010)). The major drawback to closed-reference OTU
62   picking, however, is that it cannot identify novel diversity: if a sequence has no match in the
63   reference database, it cannot be included in the analysis, restricting analyses to already-known
64   taxa. (Of course, the importance of this limitation decreases as the reference database increases in
65   coverage.)

66   Finally, open-reference OTU picking combines the previous protocols. First, input sequences are
67   clustered against a reference database in parallel in a closed-reference OTU picking process.
68   However, rather than discarding sequences that fail to match the reference, these "failures" are
69   clustered *de novo* in a serial process. Open-reference OTU picking offers benefits over both the
70   *de novo* and closed-reference protocols. Because it includes the parallel closed-reference step, it
71   will typically run faster than *de novo* OTU picking. And, since it includes *de novo* OTU picking
72   of the sequences that fail to hit the reference database, all sequences are clustered, so analyses are
73   not restricted to already-known OTUs. However, because the *de novo* clustering process is run
74   serially, it can still be prohibitively slow for very large datasets or datasets with a substantial
75   number of sequences that fail to hit the reference database. Because of these long runtimes, it has
76   not yet been widely applied despite the benefits it offers.

77   We present a novel strategy for open-reference OTU picking that allows a larger portion of the
78   computation to be run in parallel, which we call *subsampled open-reference OTU picking*,
79   allowing open-reference OTU picking on very large datasets. We compare this method to

80 "classic" open-reference OTU picking (as described in the previous paragraph) to confirm that,
81 despite potentially slightly different OTU definitions, the summary statistics that are often used
82 derive biological conclusions from application of these different methods to the same data set
83 would remain the same. To achieve this, we show that alpha diversity, beta diversity, and
84 taxonomic profiles are highly correlated between the "classic" open-reference OTU picking and
85 subsampled open-reference OTU picking. We also compare these methods to *de novo* and closed-
86 reference OTU picking, and explore the effect of dataset and algorithm parameters on runtime
87 and analysis results. We note that we specifically focus on centroid-based greedy clustering
88 approaches in this study (e.g., as in uclust and cd-hit (Li and Godzik 2006; Edgar 2010)), not
89 approaches that require alignment of all pairs of unique sequences (i.e., the hierarchical methods
90 described in (Schloss and Westcott 2011)), as the former scale better to larger data sets. However,
91 because our full evaluation framework (metrics and data sets) and the EMP raw sequence data are
92 all freely accessible, it is straight-forward for other groups to reproduce these evaluations on
93 alternative methods.

94 All analyses presented here are performed using the QIIME and pandas python packages. As far
95 as we know, QIIME contains the only existing implementation of the subsampled open-reference
96 OTU picking algorithm, but the algorithm is not QIIME-specific. Thus while our comparison is
97 based on specific QIIME/uclust-based implementations of *de novo*, *closed reference*, *classic*
98 *open reference,* and *subsampled open reference* OTU picking, our findings should be general to
99 other implementations of these algorithms.

Materials and Methods

Subsampled open-reference OTU picking algorithm

102 Open-reference OTU picking is preferable to the other methods presented here because it
103 combines the advantages of closed-reference and *de novo* clustering. However, the *de novo* step
104 of open-reference OTU picking can only be run serially, and therefore can be time-consuming for
105 large datasets if many sequences fail to hit the reference database. To improve the runtime of
106 open-reference OTU picking, we developed *subsampled open-reference OTU picking*, which
107 incrementally increases the size of the reference database by *de novo* clustering a subset of the
108 sequences that fail to match the reference database. The remainder of the sequences that fail to hit
109 the reference database can then be clustered against these new cluster centroids in a parallel
110 closed-reference OTU picking process. This allows for partial parallelization of the *de novo*
111 clustering step and can significantly decrease runtime on large datasets, allowing open-reference
112 OTU picking to scale to billions of input sequences (e.g., as generated in multiple Illumina HiSeq
113 2000 runs). It can additionally be run iteratively, so that representative sequences for the new
114 (i.e., non-reference) OTUs can be combined with the reference database for future OTU picking
115 runs. It is important to note that runtime is not always reduced with subsampled open-reference
116 OTU picking. Data set and algorithm parameters have a large effect on runtime (discussed further
117 in *Runtime differences*). This approach is similar to the Buckshot algorithm (Cutting et al. 1992;
118 Jensen et al. 2002), initially described for semantic clustering of documents in a corpus, though

119   we do not use the parallel hierarchical clustering approach described by (Jensen et al. 2002) for

120   initial clustering definition.

121   A detailed description of this workflow is illustrated in Figure 1. It is implemented using uclust

122   v1.2.22q (Edgar 2010) for clustering in QIIME 1.6.0 (Caporaso et al. 2010) and later, though any

123   sequence clustering software that provides support for *de novo* and closed-reference clustering

124   could be substituted for uclust in an alternate implementation. The inputs provided to this method

125   are demultiplexed, quality-filtered sequences, and a reference sequence collection (for example,

126   the Greengenes 13_8 97% OTU representative sequences (DeSantis et al. 2006; McDonald,

127   Price, et al. 2012)). First, sequences are clustered in parallel using a closed-reference OTU

128   picking workflow, where sequences are queried against the reference database at percent identity

129   *s* (default 97%). If a read matches a reference sequence at greater than or equal to *s%* identity, it

130   is assigned to the OTU defined by that reference sequence. These are referred to as the reference

131   OTUs. Next, a random subsample of *n%* (*n* should be small, the default value in QIIME 1.8.0-

132   dev and earlier is 0.1%) of the sequences that failed to match the reference sequence collection

133   are clustered *de novo*, and the cluster centroids for all resulting OTUs are used to define a new

134   reference sequence collection. Those OTUs are referred to as the new reference OTUs. The

135   sequences that were not included in the random subsample that was clustered *de novo* then go

136   through an additional round of parallel closed-reference OTU picking, this time where they are

137   clustered against the new reference OTUs based on matching a sequence in the new reference

138   sequence collection at greater than or equal to *s%* identity. This creation of a "new reference

139   database" allows us to harness the parallelization of our closed-reference OTU picking pipeline,

140   greatly decreasing the time it takes for sequences that fail to hit the initial reference database to

141   be clustered into OTUs. In the final clustering step, sequences that fail to hit a reference sequence

142   during this final closed-reference OTU picking step are clustered *de novo*. These are referred to

143   as the clean-up OTUs. Finally, the reference OTUs, new reference OTUs, and clean-up OTUs are

144   combined into a single OTU table (i.e., table of counts of OTUs on a per-sample basis, as

145   described in (McDonald, Clemente, et al. 2012)), and this table, as well as a filtered table

146   excluding OTUs with counts less than or equal to a user-defined threshold *c*, are provided to the

147   user. By default, *c=2*, so each OTU is observed at least twice (i.e., singleton OTUs are excluded).

148   Because many more of the sequences can be clustered using closed-reference OTU picking in

149   this workflow, it can run in far less time than classic open-reference OTU picking (see *Runtime*

150   *Differences* section below).

151   **Evaluation of subsampled open-reference OTU picking**

152   We validated the subsampled open-reference OTU picking workflow by comparing it to *de novo*,

153   closed-reference, and classic (i.e., non subsampled) open-reference clustering methods on three

154   different datasets: the Lauber "88 Soils" study (Lauber et al. 2009) (referred to as *88-soils* here),

155   the Caporaso "Moving Pictures" study (Caporaso et al. 2011) (referred to as *moving-pictures*

156   here), and the Costello "Whole Body" study (Costello et al. 2009) (referred to as *whole-body*

157   here) using three metrics. Table 1 provides a description of the OTU picking methods being

158   compared. First, we tested the correlation between sample alpha diversities (OTU counts, i.e.

159   QIIME's *observed species* metric, and Phylogenetic Diversity (PD) (Faith 1992)) based on

160 subsampled open-reference OTU picking and the other OTU picking protocols. Next, we tested
161 whether beta diversity patterns (as determined by weighted and unweighted UniFrac (Lozupone
162 and Knight 2005) distances between samples) were consistent across OTU picking protocols,
163 based on Mantel tests (Mantel 1967) with 1000 Monte Carlo iterations. Finally, we tested
164 whether the same taxonomic profiles were obtained on a per-sample basis using each of the OTU
165 picking methods. It is important to note that we are not trying to assess whether one method is
166 better than another using these metrics. Instead, we are testing whether the methods give highly
167 correlated results.

Data availability

169 The raw sequence data analyzed in this study is available in the QIIME Database under accession
170 numbers 103 (88-soils), 449 (whole-body), and 550 (moving-pictures). All analyses were run
171 with QIIME 1.8.0-dev. All commands, as well as all processed data and IPython Notebooks that
172 illustrate how to work with that data are available in this project's GitHub repository at
173 https://github.com/gregcaporaso/cloaked-octo-ninja.

Results and Discussion

Subsampled versus "classic" open-reference OTU picking

176 Alpha diversity (Table 2; whole-body PD Pearson r=0.989; 88-soils PD Pearson r=0.930;
177 moving-pictures PD Pearson r=0.996), beta diversity (Table 3; whole-body unweighted UniFrac
178 Mantel r=0.948; 88-soils unweighted UniFrac Mantel r=0.939; moving-pictures unweighted
179 UniFrac Mantel r=0.991) and taxonomic summaries (Table 4; whole-body: r=0.999 at phylum
180 level, 0.999 at species level; 88-soils r=0.999 at phylum level, r=0.999 at species level; moving-
181 pictures r=0.999 at phylum level, r=0.999 at species level) were highly correlated between classic
182 and subsampled open-reference OTU picking. Minor differences likely arise from the non-
183 deterministic step of rarefying all samples to even sampling depth before comparing samples.
184 These results suggest that subsampled open-reference picking yields the same results as classic
185 open-reference OTU picking, including identical numbers of sequences failing to hit the
186 reference database, and therefore is a suitable replacement.

Application to the Earth Microbiome Project dataset

188 In order to evaluate the effectiveness of the subsampled open-reference OTU picking method on
189 an extremely large data set, the first 15,000 samples (1.3 billion V4 16S rRNA amplicons) from
190 the Earth Microbiome Project (EMP, (Gilbert et al. 2010)) were processed on the Amazon Web
191 Services (AWS) EC2 platform. These samples were split across more than 60 studies, which were
192 clustered iteratively. To the best of our knowledge, this is the largest OTU picking run ever
193 completed. We created a StarCluster-based (http://star.mit.edu/cluster/) virtual cluster on AWS
194 using between 8 and 18 M2.4xlarge spot instances (the number of instances was varied at
195 different stages of the run). Each instance (or virtual cluster node) had 69 GB RAM and 8 cores.
196 A total of 11,242 CPU hours were consumed to complete subsampled open-reference OTU
197 picking (at 97% nucleotide identity), and the combined input and output files consumed 1.2 TB
198 of disk space. (This runtime includes the pre-filtering step. The process would have completed

199 much faster if this were disabled.) The resulting OTU table contained 5.6 million non-singleton

200 OTUs. This is the largest number of OTUs identified, and the most comprehensive survey of

201 microbial diversity across environment types to date, so it likely suggests the magnitude of the

202 lower-bound on the microbial diversity of the Earth (although the accuracy is limited because

203 some of these OTUs may be artifacts of PCR or sequencing: such artifacts, e.g. chimeras, need to

204 be identified after the OTU picking step).

205 We were next interested in how long the de novo clustering step of classic open-reference OTU

206 picking would take on the EMP data set, but as we'll illustrate this is an intractable problem in

207 practice with current computer hardware. We began by applying de novo clustering using the

208 "fast" uclust parameter settings to the representative sequences from the 5.6 million non-

209 singleton OTUs from the run described above. These representative sequences represent the full

210 alpha diversity of the EMP data set (a property known to be important to runtime of de novo and

211 open reference OTU clustering) but the data set contains only 5.6m sequences, so is feasible to

212 cluster de novo. We then subsampled this to contain between 10% and 80% of those sequences,

213 in steps of 10% with 10 iterations at each step, and compiled the runtime for each clustering run.

214 Figure 2 illustrates the relationship between runtime and input sequence count, along with the

215 results of a regression analysis presenting median runtime as a function of sequence count

216 (r^2=0.98, p=8e-6).

217 In the subsampled open-reference OTU picking run on the EMP dataset, 660 million sequences

218 failed to hit the reference database, and therefore need to be clustered de novo clustering in open-

219 reference OTU picking. While it is obviously problematic to use a regression model trained on

220 5.6 million sequences to extrapolate the runtime on 660 million sequences, we feel that this can

221 give us an idea of the magnitude of the runtime for the serial de novo clustering of the full

222 dataset. Our regression model projects that the serial de novo clustering of sequences that fail to

223 hit the reference data set would require approximately 150 days to run (in wall time). In contrast,

224 the subsampled open-reference OTU picking run presented here (which included the pre-filtering

225 step) ran in just under 30 days of wall time. This illustrates that while on relatively small data sets

226 the performance enhancement of subsampled relative to classic open-reference OTU picking is

227 either non-existence or modest (discussed in *Run-time differences*), on datasets at the current

228 upper limit of size, the increased parallelizability of subsampled open-reference OTU picking

229 makes open-reference OTU picking far more tractable.

230 **Run-time differences**
231 The speed improvements of subsampled open-reference OTU picking arise from the fact that a

232 larger portion of the clustering process can be parallelized. When not run in parallel, or run in

233 parallel over only a few (e.g., 3) CPUs, classic open-reference OTU picking is likely to be faster.

234 Similarly, for smaller data sets (e.g., less than a few million sequences), especially if most

235 sequences have a match in the reference database (e.g., with human gut microbiome data), classic

236 open-reference OTU picking will achieve similar runtimes to subsampled open-reference

237 clustering (Table 5). However, in these cases, the results are still highly correlated, so if in doubt

238 of which method will be faster, subsampled open-reference OTU picking is a reasonable choice

239   as the summary statistics of interest (often alpha diversity, beta diversity and taxonomic profiles)
240   are very unlikely to be different between the two methods.

241   When more sequences fail to hit the reference database, subsampled open-reference OTU picking
242   becomes faster than classic open-reference OTU picking (Table 6). To illustrate this, we clustered
243   the moving-pictures sequences against the 82% and 97% Greengenes reference OTUs at 97%
244   identity using subsampled and classic open-reference OTU picking on 29 processors. When
245   clustering against the 82% OTUs, 52.1 million failed to hit the reference, while when clustering
246   against the 97% OTUs 3.4 million sequences failed to hit the reference. Subsampled open-
247   reference OTU picking ran in 4000s less wall time than classic open-reference clustering (in a
248   single run of each on a system dedicated for this run time comparison) against the 82% OTUs,
249   and in 72s less time against the 97% OTUs, illustrating that as more sequences fail to hit the
250   reference, subsampled open-reference OTU picking offers more of an advantage. This runtime
251   difference would be even larger if the job were split over more processors.

252   Another parameter that can affect runtime of subsampled open-reference OTU picking is the size
253   of the random subsample that is selected. The optimal setting for this parameter is affected by the
254   size of the dataset being clustered and the diversity of the sequences that fail to match the
255   reference database. On small datasets, or datasets with a lot of novel diversity, a large fraction
256   (e.g., 1%) is better than a small fraction (e.g., 0.001%), but as the data set increases in size a large
257   fraction can result in far more time spent performing *de novo* clustering of the sequences that
258   initially fail to hit the reference database. We recommend using the default (0.1% in QIIME
259   1.8.0-dev and earlier), which was chosen to reduce runtime on larger datasets where optimized
260   runtime is more important. As this parameter setting approaches zero, subsampled open-reference
261   OTU picking becomes more like classic open-reference OTU picking, in that more of the reads
262   that fail to hit the reference database are clustered de novo serially, and at the limit of 0% of
263   sequences subsampled, subsampled open reference OTU picking becomes classic open-reference
264   OTU picking. The summary statistics investigated here are highly correlated between classic and
265   subsampled open-reference OTU picking, suggesting that this parameter setting will not affect
266   those statistics, but can affect runtime.

267   **Pre-filtering**
268   QIIME's open-reference OTU picking workflow optionally includes a pre-filtering step, where
269   sequences are searched against the reference database with low percent identity (the default in
270   QIIME 1.8.0 and earlier is 60%), and sequences that fail to match are discarded from the
271   analysis. The goal of this process is to discard sequences that are likely not representatives of the
272   marker gene, such as host genomic sequences or products of non-specific amplification. This
273   process is functionally similar to closed-reference OTU picking (sequence reads are searched
274   against a pre-defined reference database), and therefore is easily run in parallel.

275   We show that alpha diversity (Table 2; whole-body PD Pearson r=0.991; 88-soils PD Pearson
276   r=0.930; moving-pictures PD Pearson r=0.996), beta diversity (Table 3; whole-body unweighted
277   UniFrac Mantel r=0.953; 88-soils unweighted UniFrac Mantel r=0.940; moving-pictures

278 unweighted UniFrac Mantel r=0.990) and taxonomic summaries (Table 4; whole-body: r=1.000
279 at phylum level, r=1.000 at species level; 88-soils r=1.000 at phylum level, r=1.000 at species
280 level; moving-pictures r=1.000 at phylum level, r=0.999 at species level) are highly correlated
281 between the pre-filtered and non-pre-filtered results, when pre-filtering is performed at percent
282 identity of 60%. Despite nearly identical results, the pre-filtering process results in vastly
283 increased runtimes. Consequently, we no longer recommend pre-filtering of sequences prior to
284 open-reference OTU picking. Rather, contaminant sequences should be discarded after OTU
285 picking. This feature is now disabled by default starting with QIIME 1.8.0-dev.

286 One case where pre-filtering may prove useful is in the preparation of sequence data where there
287 is a large amount of contamination of non-marker-gene sequence, for example host genomic
288 contamination. In this case, pre-filtering can be useful to remove those sequences prior to
289 clustering. Note that if you suspect that your sample may contain human genomic contaminant
290 sequences, it is important to filter them out before analysis or data deposition due to Institutional
291 Review Board or other ethical concerns related to release of human DNA sequences.

Clustering parameters

293 We also investigated the effect of clustering parameters on the same summary statistics, as these
294 can have a considerable effect on runtime. We compared uclust's default settings (referred to in
295 QIIME as "fast mode") with the default settings in QIIME 1.8.0 and earlier ("slow mode"). We
296 again compared the methods based on the degree to which they resulted in correlated alpha
297 diversity (Table 2), beta diversity (Table 3), and taxonomic results (Table 4), and found that all
298 results were highly correlated between fast and slow modes. This suggests that while fast mode
299 will occasionally make suboptimal OTU assignments, the effects are subtle enough to be
300 unnoticeable in downstream ecological analyses. We therefore recommend using the "fast"
301 settings for decreased runtime, and these are now the default in QIIME 1.8.0-dev.

302 We do recommend using the "slow" settings if clustering sequences to build reference OTUs (for
303 example, as is performed when building the Greengenes reference OTU collection (McDonald,
304 Price, et al. 2012)) because suboptimal OTU assignments can have further reaching
305 consequences. For example, "splitting" an OTU (i.e., defining two sequences that are within *s%*
306 identity of each other as the centroids of two different *s%* OTUs), which is always a possibility in
307 greedy clustering algorithms, is more common with the "fast" settings than with the "slow"
308 settings. If this occurs in a single study, the downstream effects are limited to that study and are
309 likely only to be problematic if the split OTU is of key significance to the system being
310 investigated. However, a split OTU when defining reference OTUs is more problematic, because
311 those definitions will be used in many studies, increasing the chance that the split OTU will be
312 problematic for someone. For this application, the processing step is typically only run once per
313 database release (which is relatively infrequent). Therefore, the longer runtime is preferable to
314 less accurate OTU definitions in this particular application. If splitting and lumping of OTUs is of
315 concern on your dataset, you may want to experiment with the "slow" parameter settings, which
316 are still accessible in QIIME and we also recommend exploring the use of Oligotyping (Eren et
317 al. 2013).

318 **Consistent OTU definitions across runs: iterative open-reference OTU picking**

319 Subsampled open-reference clustering, as implemented in QIIME, provides new identifiers for
320 sequences that fail to match the reference database, allowing OTUs to be directly compared
321 across clustering runs (although sequences clustered against this expanded reference sequence
322 collection do need to be from the same gene fragment as the sequences used to expand the
323 reference sequence collection). These OTUs can also be used in iterative OTU picking, which is
324 useful in studies where sequence data is continuously accumulating, for example in routine
325 monitoring of microbial communities in human subjects (e.g. patients monitored over time), the
326 built-environment, or during environmental clean-up.

Conclusions

328 Taken together, the reduced runtime of subsampled open-reference OTU picking relative to
329 classic open-reference OTU picking on large datasets, and the benefits that open-reference OTU
330 picking offers over full *de novo* OTU picking (vastly decreased runtime) and closed-reference
331 OTU picking (all sequences are clustered, not only those that match the reference collection), we
332 recommend subsampled open-reference OTU picking when a reference collection is available.

333 Because the metrics provided here show that the same summary statistics are derived from the
334 four OTU picking protocols, an interesting question is whether *de novo* or open-reference OTU
335 picking offers any benefit over closed-reference OTU picking. The primary motivation for using
336 methods that incorporate previously unknown OTUs (i.e., those that are not represented in the
337 reference database) such as *de novo* and open-reference OTU picking is that OTUs not
338 represented in the reference database might best illustrate a biological pattern of interest. For
339 example, in the 88-soils data analyzed here, 1 of the top 10 OTUs identified as significantly
340 different across sample pH is an OTU that is not represented in the reference database (Table 8)
341 (this OTU was classified as in the *Actinomycetales* order by QIIME's uclust-based taxonomy
342 classifier). Similarly, for the whole-body data set, 2 of the top 10 OTUs identified as significantly
343 different across body sites were not represented in the reference database (these were classified as
344 *Prevotella melaninogenica* and *Veillonella parvula* by QIIME's uclust-based taxonomy
345 classifier). On the other hand, in the moving-pictures data analyzed here, all of the top 10 OTUs
346 identified as significantly different across body site were OTUs represented in the reference
347 database. Table 7 illustrates the fraction of OTUs not represented in the reference database by
348 environment based on the Earth Microbiome Project dataset. We expect that using OTU picking
349 methods that incorporate new OTUs is more important in samples where this fraction is higher.

350 In conclusion, this paper presents the performance-optimized subsampled open-reference OTU
351 picking algorithm, now available in QIIME. This method can be applied iteratively to define
352 stable OTUs across sequencing runs, and achieves nearly identical results to "classic" open-
353 reference OTU picking (i.e., not including the subsampling step). It enables massive sequencing
354 projects such as the Earth Microbiome Project to use open-reference OTU picking in far less time
355 than is possible with classic open-reference OTU picking, which will facilitate our exploration of
356 microbial diversity. Further, the iterative nature of the process (which is also possible with classic

357 open-reference OTU picking) enables progressively expanding datasets, as might be generated in
358 clinical laboratories as microbiome-based medical treatment becomes a reality, to cluster OTUs
359 using OTU definitions from previous clustering runs as reference sequences. This avoids re-
360 clustering all sequences every time new sequences are generated, thereby vastly decreasing
361 computational costs.

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365 (www.earthmicrobiome.org) and all amplicon and metadata has been made public through the
366 data portal (www.microbio.me/emp).

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Figure 1

Schematic of the subsampled open-reference OTU picking algorithm.

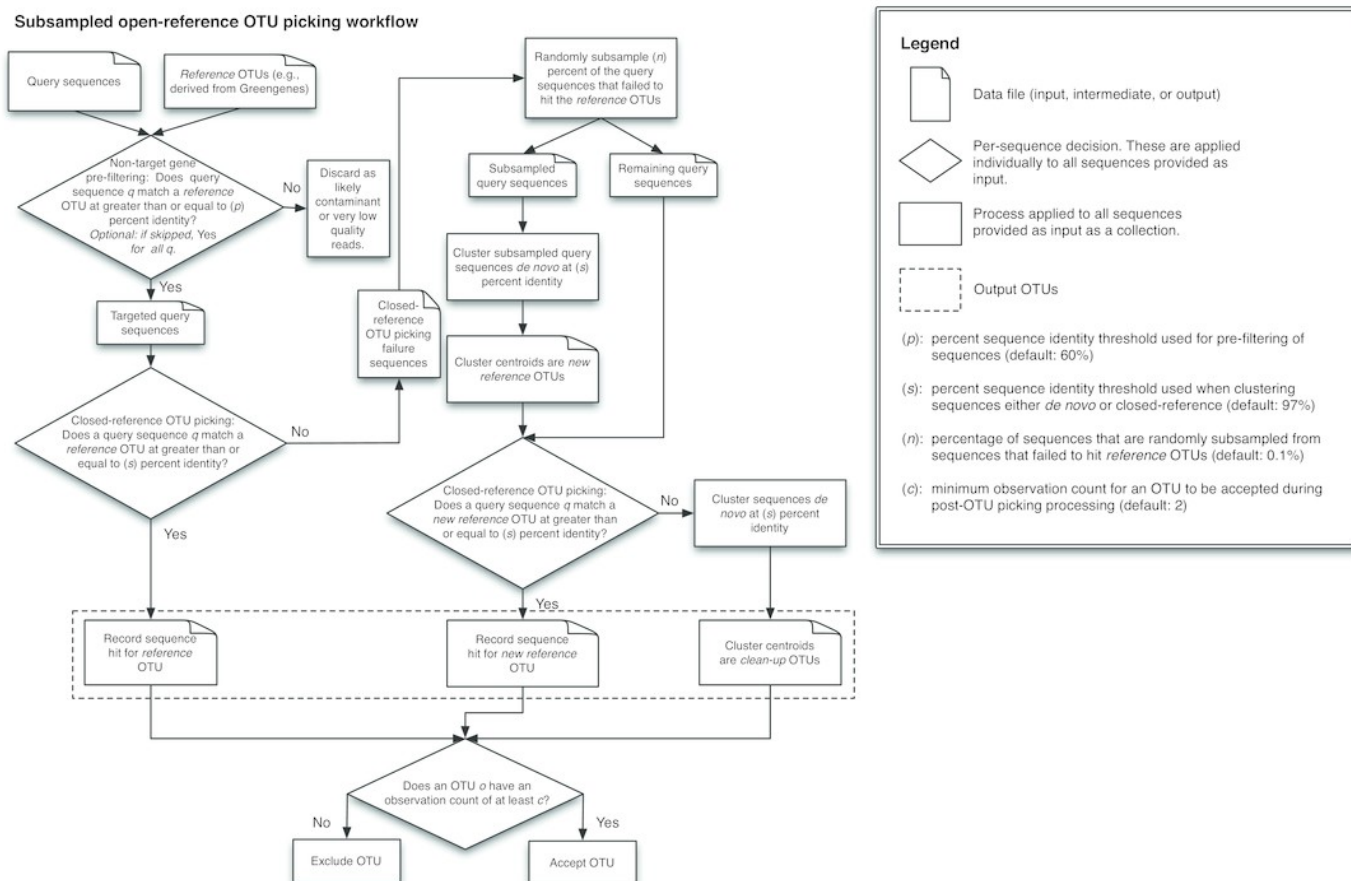
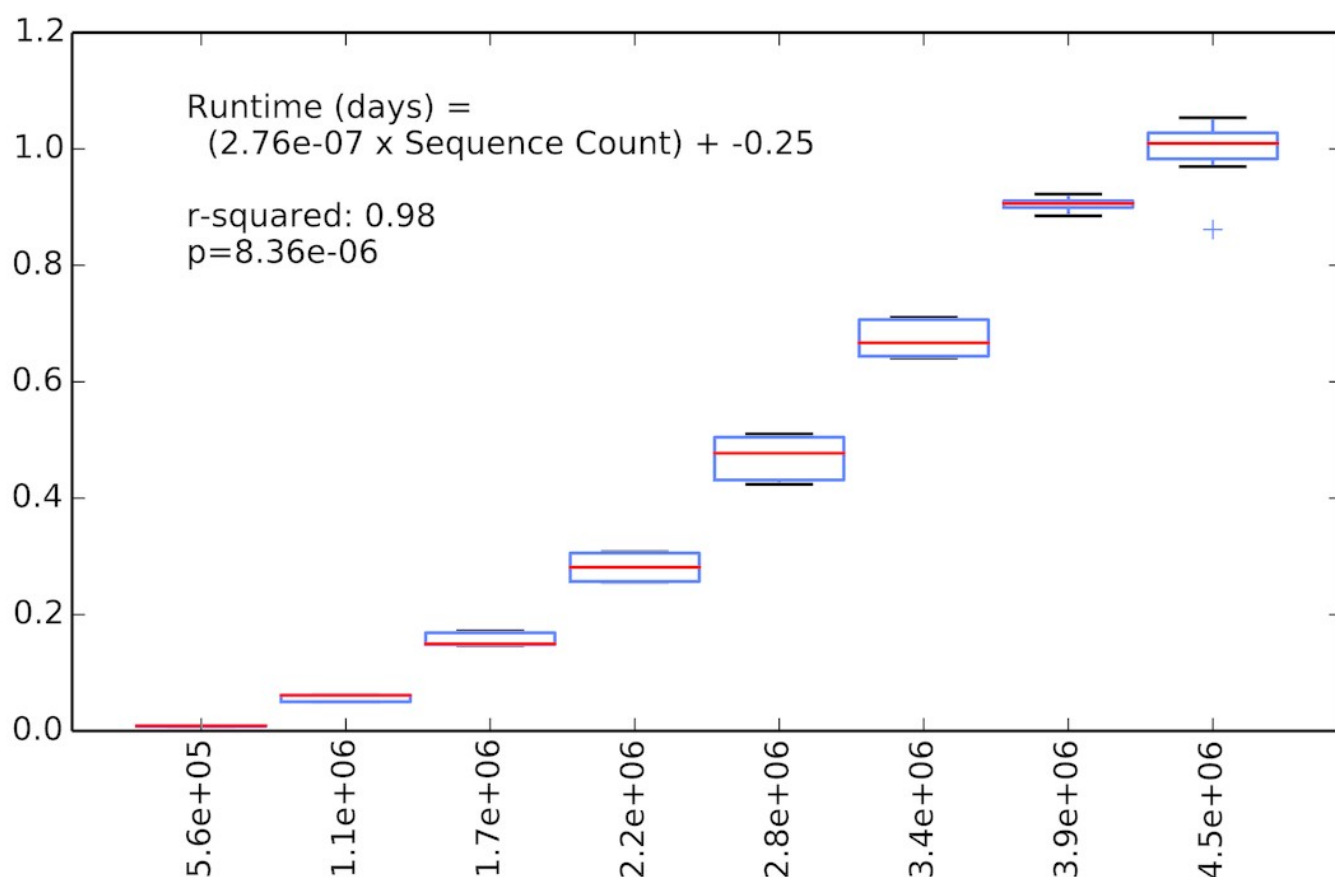



Figure 2

Runtime comparison.

Runtime of de novo clustering using "fast" uclust parameters versus number of sequences to be clustered, where sequences are obtained from the EMP subsampled open-reference OTU picking run.

Method definitions.

Definitions of the OTU picking methods being compared here, based on the abbreviations used throughout the paper. From here, we refer to each method by its abbreviation for simplicity. We note that the both de novo (uc) and classic open-reference OTU picking (ucr) are accessed through QIIME's pick_de_novo_otus.py command. ucr is applied when pick_otus:otu_picking_method uclust_ref is specified in the parameters file, and uc is applied when that option is absent. The exact command/parameter combinations used for each OTU picking run are provided in the study's GitHub repository (see Data Availability).

abbreviation	title	command	max_accepts	max_rejects	stepwords	wordlength	prefilter_percent_id	min_otu_size	speed_mode	processors	reference_percent_id	subsample_fraction
uc	De novo	pick_de_novo_otus.py	20	500	20	12	NA	NA	slow	1	0.97	NA
ucr	Legacy open reference	pick_de_novo_otus.py	20	500	20	12	NA	NA	slow	10	0.97	NA
ucrC	Closed reference	pick_closed_reference_otus.py	20	500	20	12	NA	NA	slow	10	0.97	NA
ucrss	Subsampled open reference	pick_open_reference_otus.py	20	500	20	12	0	1	slow	10	0.97	0.001
ucrss_wfilter	Subsampled open reference, filtered	pick_open_reference_otus.py	20	500	20	12	0.6	1	slow	10	0.97	0.001
uc_fast	De novo, fast settings	pick_de_novo_otus.py	1	8	8	8	NA	NA	fast	1	0.97	NA
ucr_fast	Legacy open reference, fast settings	pick_de_novo_otus.py	1	8	8	8	NA	NA	fast	10	0.97	NA
ucrC_fast	Closed reference, fast settings	pick_closed_reference_otus.py	1	8	8	8	NA	NA	fast	10	0.97	NA
ucrss_fast	Subsampled open reference, fast settings	pick_open_reference_otus.py	1	8	8	8	0	1	fast	10	0.97	0.001
ucrss_wfilter_fast	Subsampled open reference, filtered, fast settings	pick_open_reference_otus.py	1	8	8	8	0.6	1	fast	10	0.97	0.001
ucr_fast_O29_r82	Legacy open reference, fast settings, 82% reference OTUs, 29 processors	pick_de_novo_otus.py	1	8	8	8	0	1	fast	29	0.82	0.001
ucr_fast_O29_r97	Legacy open reference, fast settings, 29 processors	pick_de_novo_otus.py	1	8	8	8	0	1	fast	29	0.97	0.001
ucrss_fast_O29_r82	Subsampled open reference, fast settings, 82% reference OTUs, 29 processors	pick_open_reference_otus.py	1	8	8	8	0	1	fast	29	0.82	0.001
ucrss_fast_O29_r97	Subsampled open reference, fast settings, 29 processors	pick_open_reference_otus.py	1	8	8	8	0	1	fast	29	0.97	0.001
ucrss_fast_O29_s1	Subsampled open reference, fast settings, 29 processors, 1% subsample	pick_open_reference_otus.py	1	8	8	8	0	1	fast	29	0.97	0.1

Table 2_(on next page)

Alpha diversity results.

Pearson correlation coefficients (r) of alpha diversity for (a) 88-soils PD, (b) moving-pictures PD, (c) whole-body PD, (d) 88-soils observed species, (e) moving-pictures observed species, and (f) moving-pictures observed species.

(a)

	uc	ucr	ucrC	ucrss	ucrss_wfilter	uc_fast	ucr_fast	ucrC_fast	ucrss_fast	ucrss_fast_wfilter
uc	1	0.951	0.933	0.934	0.953	0.956	0.936	0.927	0.948	0.947
ucr	0.951	1	0.902	0.931	0.93	0.946	0.94	0.903	0.952	0.944
ucrC	0.933	0.902	1	0.894	0.909	0.905	0.914	0.978	0.902	0.911
ucrss	0.934	0.931	0.894	1	0.929	0.944	0.935	0.894	0.948	0.949
ucrss_wfilter	0.953	0.93	0.909	0.929	1	0.952	0.933	0.903	0.931	0.943
uc_fast	0.956	0.946	0.905	0.944	0.952	1	0.953	0.898	0.956	0.96
ucr_fast	0.936	0.94	0.914	0.935	0.933	0.953	1	0.914	0.95	0.952
ucrC_fast	0.927	0.903	0.978	0.894	0.903	0.898	0.914	1	0.902	0.903
ucrss_fast	0.948	0.952	0.902	0.948	0.931	0.956	0.95	0.902	1	0.962
ucrss_fast_wfilter	0.947	0.944	0.911	0.949	0.943	0.96	0.952	0.903	0.962	1

(b)

	uc	ucr	ucrC	ucrss	ucrss_wfilter	uc_fast	ucr_fast	ucrC_fast	ucrss_fast	ucrss_fast_wfilter
uc	1	0.996	0.993	0.996	0.996	0.995	0.996	0.992	0.996	0.996
ucr	0.996	1	0.993	0.997	0.997	0.995	0.996	0.992	0.996	0.997
ucrC	0.993	0.993	1	0.994	0.991	0.994	0.994	0.998	0.995	0.994
ucrss	0.996	0.997	0.994	1	0.996	0.996	0.997	0.994	0.997	0.997
ucrss_wfilter	0.996	0.997	0.991	0.996	1	0.994	0.995	0.991	0.996	0.996
uc_fast	0.995	0.995	0.994	0.996	0.994	1	0.997	0.994	0.997	0.996
ucr_fast	0.996	0.996	0.994	0.997	0.995	0.997	1	0.994	0.997	0.997
ucrC_fast	0.992	0.992	0.998	0.994	0.991	0.994	0.994	1	0.994	0.994
ucrss_fast	0.996	0.996	0.995	0.997	0.996	0.997	0.997	0.994	1	0.997
ucrss_fast_wfilter	0.996	0.997	0.994	0.997	0.996	0.996	0.997	0.994	0.997	1

(c)

	uc	ucr	ucrC	ucrss	ucrss_wfilter	uc_fast	ucr_fast	ucrC_fast	ucrss_fast	ucrss_fast_wfilter
uc	1	0.985	0.957	0.985	0.985	0.984	0.986	0.961	0.983	0.984
ucr	0.985	1	0.956	0.99	0.989	0.988	0.987	0.96	0.987	0.986
ucrC	0.957	0.956	1	0.961	0.958	0.959	0.961	0.99	0.953	0.961
ucrss	0.985	0.99	0.961	1	0.991	0.988	0.99	0.964	0.989	0.987
ucrss_wfilter	0.985	0.989	0.958	0.991	1	0.985	0.989	0.963	0.987	0.985
uc_fast	0.984	0.988	0.959	0.988	0.985	1	0.986	0.961	0.986	0.985
ucr_fast	0.986	0.987	0.961	0.99	0.989	0.986	1	0.965	0.988	0.989
ucrC_fast	0.961	0.96	0.99	0.964	0.963	0.961	0.965	1	0.957	0.965
ucrss_fast	0.983	0.987	0.953	0.989	0.987	0.986	0.988	0.957	1	0.986
ucrss_fast_wfilter	0.984	0.986	0.961	0.987	0.985	0.985	0.989	0.965	0.986	1

(d)

	uc	ucr	ucrC	ucrss	ucrss_wfilter	uc_fast	ucr_fast	ucrC_fast	ucrss_fast	ucrss_fast_wfilter
uc	1	0.948	0.88	0.909	0.924	0.935	0.934	0.877	0.925	0.913
ucr	0.948	1	0.905	0.946	0.947	0.947	0.953	0.903	0.938	0.932
ucrC	0.88	0.905	1	0.926	0.888	0.882	0.908	0.973	0.91	0.896
ucrss	0.909	0.946	0.926	1	0.932	0.923	0.935	0.915	0.931	0.929
ucrss_wfilter	0.924	0.947	0.888	0.932	1	0.943	0.946	0.884	0.932	0.927
uc_fast	0.935	0.947	0.882	0.923	0.943	1	0.942	0.883	0.941	0.94
ucr_fast	0.934	0.953	0.908	0.935	0.946	0.942	1	0.908	0.943	0.932
ucrC_fast	0.877	0.903	0.973	0.915	0.884	0.883	0.908	1	0.904	0.906
ucrss_fast	0.925	0.938	0.91	0.931	0.932	0.941	0.943	0.904	1	0.953
ucrss_fast_wfilter	0.913	0.932	0.896	0.929	0.927	0.94	0.932	0.906	0.953	1

(e)

	uc	ucr	ucrC	ucrss	ucrss_wfilter	uc_fast	ucr_fast	ucrC_fast	ucrss_fast	ucrss_fast_wfilter
uc	1	0.992	0.984	0.992	0.992	0.989	0.99	0.978	0.989	0.99
ucr	0.992	1	0.994	0.998	0.998	0.992	0.997	0.991	0.997	0.997
ucrC	0.984	0.994	1	0.995	0.995	0.984	0.993	0.997	0.994	0.994
ucrss	0.992	0.998	0.995	1	0.998	0.992	0.997	0.991	0.997	0.997
ucrss_wfilter	0.992	0.998	0.995	0.998	1	0.992	0.997	0.991	0.997	0.997
uc_fast	0.989	0.992	0.984	0.992	0.992	1	0.993	0.981	0.992	0.992
ucr_fast	0.99	0.997	0.993	0.997	0.997	0.993	1	0.992	0.998	0.998
ucrC_fast	0.978	0.991	0.997	0.991	0.991	0.981	0.992	1	0.993	0.992
ucrss_fast	0.989	0.997	0.994	0.997	0.997	0.992	0.998	0.993	1	0.998
ucrss_fast_wfilter	0.99	0.997	0.994	0.997	0.997	0.992	0.998	0.992	0.998	1

(f)

	uc	ucr	ucrC	ucrss	ucrss_wfilter	uc_fast	ucr_fast	ucrC_fast	ucrss_fast	ucrss_fast_wfilter
uc	1	0.986	0.971	0.986	0.986	0.993	0.988	0.972	0.988	0.987
ucr	0.986	1	0.984	0.995	0.995	0.987	0.993	0.98	0.993	0.993
ucrC	0.971	0.984	1	0.985	0.984	0.97	0.981	0.992	0.98	0.979
ucrss	0.986	0.995	0.985	1	0.995	0.987	0.993	0.981	0.993	0.992
ucrss_wfilter	0.986	0.995	0.984	0.995	1	0.986	0.993	0.979	0.992	0.992
uc_fast	0.993	0.987	0.97	0.987	0.986	1	0.989	0.972	0.99	0.988

ucr_fast	0.988	0.993	0.981	0.993	0.993	0.989	1	0.981	0.994	0.994
ucrC_fast	0.972	0.98	0.992	0.981	0.979	0.972	0.981	1	0.982	0.979
ucrss_fast	0.988	0.993	0.98	0.993	0.992	0.99	0.994	0.982	1	0.995
ucrss_fast_w filter	0.987	0.993	0.979	0.992	0.992	0.988	0.994	0.979	0.995	1

Beta diversity results

Mantel correlation coefficients (r) of beta diversity for (a) 88-soils unweighted UniFrac, (b) moving-pictures unweighted UniFrac, (c) whole-body unweighted UniFrac, (d) 88-soils weighted UniFrac, (e) moving-pictures weighted UniFrac, and (f) moving-pictures weighted UniFrac.

(a)

	uc	ucr	ucrC	ucrss	ucrss_wfilter	uc_fast	ucr_fast	ucrC_fast	ucrss_fast	ucrss_fast_wfilter
uc	NA	0.935	0.908	0.944	0.942	0.939	0.945	0.909	0.943	0.941
ucr	NA	NA	0.915	0.94	0.945	0.934	0.942	0.918	0.944	0.949
ucrC	NA	NA	NA	0.917	0.91	0.926	0.913	0.95	0.917	0.92
ucrss	NA	NA	NA	NA	0.94	0.938	0.945	0.914	0.938	0.942
ucrss_wfilter	NA	NA	NA	NA	NA	0.934	0.943	0.907	0.942	0.941
uc_fast	NA	NA	NA	NA	NA	NA	0.938	0.92	0.939	0.941
ucr_fast	NA	NA	NA	NA	NA	NA	NA	0.909	0.946	0.947
ucrC_fast	NA	NA	NA	NA	NA	NA	NA	NA	0.917	0.924
ucrss_fast	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.945
ucrss_fast_wfilter	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

(b)

	uc	ucr	ucrC	ucrss	ucrss_wfilter	uc_fast	ucr_fast	ucrC_fast	ucrss_fast	ucrss_fast_wfilter
uc	NA	0.992	0.974	0.988	0.988	0.992	0.991	0.977	0.991	0.992
ucr	NA	NA	0.982	0.992	0.991	0.991	0.992	0.984	0.993	0.993
ucrC	NA	NA	NA	0.986	0.985	0.973	0.982	0.994	0.981	0.981
ucrss	NA	NA	NA	NA	0.99	0.988	0.992	0.987	0.992	0.991
ucrss_wfilter	NA	NA	NA	NA	NA	0.986	0.99	0.986	0.99	0.991
uc_fast	NA	NA	NA	NA	NA	NA	0.991	0.976	0.992	0.991
ucr_fast	NA	NA	NA	NA	NA	NA	NA	0.983	0.993	0.992
ucrC_fast	NA	NA	NA	NA	NA	NA	NA	NA	0.982	0.983
ucrss_fast	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.993
ucrss_fast_wfilter	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

(c)

	uc	ucr	ucrC	ucrss	ucrss_wfilter	uc_fast	ucr_fast	ucrC_fast	ucrss_fast	ucrss_fast_wfilter
uc	NA	0.935	0.891	0.938	0.936	0.93	0.926	0.889	0.933	0.925
ucr	NA	NA	0.899	0.948	0.95	0.934	0.931	0.895	0.941	0.927
ucrC	NA	NA	NA	0.908	0.899	0.878	0.885	0.952	0.897	0.878
ucrss	NA	NA	NA	NA	0.953	0.938	0.936	0.905	0.945	0.928
ucrss_wfilter	NA	NA	NA	NA	NA	0.937	0.94	0.894	0.941	0.932
uc_fast	NA	NA	NA	NA	NA	NA	0.942	0.872	0.939	0.938
ucr_fast	NA	NA	NA	NA	NA	NA	NA	0.888	0.939	0.948
ucrC_fast	NA	NA	NA	NA	NA	NA	NA	NA	0.891	0.879
ucrss_fast	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.933
ucrss_fast_wfilter	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

(d)

	uc	ucr	ucrC	ucrss	ucrss_wfilter	uc_fast	ucr_fast	ucrC_fast	ucrss_fast	ucrss_fast_wfilter
uc	NA	0.896	0.936	0.951	0.901	0.925	0.937	0.924	0.956	0.902
ucr	NA	NA	0.896	0.889	0.966	0.891	0.939	0.895	0.901	0.947
ucrC	NA	NA	NA	0.919	0.914	0.906	0.928	0.984	0.931	0.896
ucrss	NA	NA	NA	NA	0.9	0.917	0.947	0.903	0.949	0.899
ucrss_wfilter	NA	NA	NA	NA	NA	0.885	0.938	0.911	0.899	0.94
uc_fast	NA	NA	NA	NA	NA	NA	0.909	0.898	0.919	0.874
ucr_fast	NA	NA	NA	NA	NA	NA	NA	0.92	0.952	0.96
ucrC_fast	NA	NA	NA	NA	NA	NA	NA	NA	0.918	0.89
ucrss_fast	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.918
ucrss_fast_wfilter	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

(e)

	uc	ucr	ucrC	ucrss	ucrss_wfilter	uc_fast	ucr_fast	ucrC_fast	ucrss_fast	ucrss_fast_wfilter
uc	NA	0.971	0.949	0.97	0.973	0.972	0.977	0.949	0.974	0.966
ucr	NA	NA	0.928	0.952	0.952	0.957	0.958	0.928	0.96	0.954
ucrC	NA	NA	NA	0.96	0.94	0.948	0.934	0.999	0.965	0.932
ucrss	NA	NA	NA	NA	0.938	0.965	0.955	0.96	0.98	0.932
ucrss_wfilter	NA	NA	NA	NA	NA	0.946	0.966	0.941	0.951	0.967
uc_fast	NA	NA	NA	NA	NA	NA	0.97	0.948	0.971	0.949
ucr_fast	NA	NA	NA	NA	NA	NA	NA	0.934	0.967	0.967
ucrC_fast	NA	NA	NA	NA	NA	NA	NA	NA	0.965	0.932
ucrss_fast	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.951
ucrss_fast_wfilter	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

(f)

	uc	ucr	ucrC	ucrss	ucrss_wfilter	uc_fast	ucr_fast	ucrC_fast	ucrss_fast	ucrss_fast_wfilter
uc	NA	0.947	0.896	0.934	0.943	0.96	0.939	0.898	0.904	0.936
ucr	NA	NA	0.9	0.924	0.95	0.951	0.92	0.904	0.871	0.944
ucrC	NA	NA	NA	0.886	0.924	0.907	0.911	0.994	0.831	0.939
ucrss	NA	NA	NA	NA	0.944	0.92	0.917	0.882	0.918	0.911
ucrss_wfilter	NA	NA	NA	NA	NA	0.933	0.918	0.926	0.897	0.932
uc_fast	NA	NA	NA	NA	NA	NA	0.955	0.909	0.889	0.966

ucr_fast	NA	NA	NA	NA	NA	NA	NA	0.91	0.936	0.951
ucrC_fast	NA	NA	NA	NA	NA	NA	NA	NA	0.83	0.94
ucrss_fast	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.866
ucrss_fast_w filter	NA	NA	NA	NA	NA	NA	NA	NA	NA	

Table 4(on next page)

Taxonomic profile results

Pearson correlation coefficients (r) of taxonomic summaries for (a) 88-soils at phylum level, (b) 88-soils at genus level, (c) moving-pictures at phylum level, (d) moving-pictures at genus level, (e) whole-body at phylum level, and (f) whole-body at genus level.

(a)

	uc	ucr	ucrC	ucrss	ucrss_wfilter	uc_fast	ucr_fast	ucrC_fast	ucrss_fast	ucrss_fast_wfilter
uc	NA	1	0.983	1	1	1	1	0.981	1	1
ucr	NA	NA	0.983	1	1	1	1	0.981	1	1
ucrC	NA	NA	NA	0.983	0.983	0.983	0.983	0.999	0.983	0.983
ucrss	NA	NA	NA	NA	1	1	1	0.981	1	1
ucrss_wfilter	NA	NA	NA	NA	NA	1	1	0.981	1	1
uc_fast	NA	NA	NA	NA	NA	NA	1	0.981	1	1
ucr_fast	NA	NA	NA	NA	NA	NA	NA	0.981	1	1
ucrC_fast	NA	NA	NA	NA	NA	NA	NA	NA	0.981	0.981
ucrss_fast	NA	NA	NA	NA	NA	NA	NA	NA	NA	1
ucrss_fast_wfilter	NA	NA	NA	NA	NA	NA	NA	NA	NA	

(b)

	uc	ucr	ucrC	ucrss	ucrss_wfilter	uc_fast	ucr_fast	ucrC_fast	ucrss_fast	ucrss_fast_wfilter
uc	NA	0.939	0.85	0.939	0.939	1	0.94	0.84	0.94	0.94
ucr	NA	NA	0.821	1	1	0.94	0.998	0.923	0.998	0.998
ucrC	NA	NA	NA	0.821	0.821	0.85	0.82	0.818	0.82	0.82
ucrss	NA	NA	NA	NA	1	0.94	0.998	0.923	0.998	0.998
ucrss_wfilter	NA	NA	NA	NA	NA	0.94	0.998	0.923	0.998	0.998
uc_fast	NA	NA	NA	NA	NA	NA	0.94	0.84	0.94	0.94
ucr_fast	NA	NA	NA	NA	NA	NA	NA	0.921	1	1
ucrC_fast	NA	NA	NA	NA	NA	NA	NA	NA	0.921	0.921
ucrss_fast	NA	NA	NA	NA	NA	NA	NA	NA	NA	1
ucrss_fast_wfilter	NA	NA	NA	NA	NA	NA	NA	NA	NA	

(c)

	uc	ucr	ucrC	ucrss	ucrss_wfilter	uc_fast	ucr_fast	ucrC_fast	ucrss_fast	ucrss_fast_wfilter
uc	NA	1	0.997	1	1	1	1	0.997	1	0.998
ucr	NA	NA	0.997	1	1	1	1	0.997	1	0.998
ucrC	NA	NA	NA	0.997	0.997	0.997	0.997	1	0.997	0.998
ucrss	NA	NA	NA	NA	1	1	1	0.997	1	0.998
ucrss_wfilter	NA	NA	NA	NA	NA	1	1	0.997	1	0.999
uc_fast	NA	NA	NA	NA	NA	NA	1	0.997	1	0.998
ucr_fast	NA	NA	NA	NA	NA	NA	NA	0.997	1	0.998
ucrC_fast	NA	NA	NA	NA	NA	NA	NA	NA	0.997	0.997
ucrss_fast	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.998
ucrss_fast_wfilter	NA	NA	NA	NA	NA	NA	NA	NA	NA	

(d)

	uc	ucr	ucrC	ucrss	ucrss_wfilter	uc_fast	ucr_fast	ucrC_fast	ucrss_fast	ucrss_fast_wfilter
uc	NA	0.964	0.929	0.964	0.963	0.999	0.923	0.882	0.923	0.92
ucr	NA	NA	0.963	1	0.999	0.967	0.954	0.923	0.954	0.951
ucrC	NA	NA	NA	0.963	0.963	0.934	0.925	0.917	0.925	0.925
ucrss	NA	NA	NA	NA	0.999	0.967	0.954	0.923	0.954	0.951
ucrss_wfilter	NA	NA	NA	NA	NA	0.966	0.953	0.923	0.953	0.952
uc_fast	NA	NA	NA	NA	NA	NA	0.927	0.887	0.927	0.924
ucr_fast	NA	NA	NA	NA	NA	NA	NA	0.885	1	0.997
ucrC_fast	NA	NA	NA	NA	NA	NA	NA	NA	0.885	0.884
ucrss_fast	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.997
ucrss_fast_wfilter	NA	NA	NA	NA	NA	NA	NA	NA	NA	

(e)

	uc	ucr	ucrC	ucrss	ucrss_wfilter	uc_fast	ucr_fast	ucrC_fast	ucrss_fast	ucrss_fast_wfilter
uc	NA	1	0.999	1	1	1	1	0.998	1	1
ucr	NA	NA	0.999	1	1	1	1	0.998	1	1
ucrC	NA	NA	NA	0.999	0.999	0.999	0.999	0.999	0.999	0.999
ucrss	NA	NA	NA	NA	1	1	1	0.998	1	1
ucrss_wfilter	NA	NA	NA	NA	NA	1	1	0.998	1	1
uc_fast	NA	NA	NA	NA	NA	NA	1	0.998	1	1
ucr_fast	NA	NA	NA	NA	NA	NA	NA	0.998	1	1
ucrC_fast	NA	NA	NA	NA	NA	NA	NA	NA	0.998	0.998
ucrss_fast	NA	NA	NA	NA	NA	NA	NA	NA	NA	1
ucrss_fast_wfilter	NA	NA	NA	NA	NA	NA	NA	NA	NA	

(f)

	uc	ucr	ucrC	ucrss	ucrss_wfilter	uc_fast	ucr_fast	ucrC_fast	ucrss_fast	ucrss_fast_wfilter
uc	NA	0.959	0.9	0.959	0.959	1	0.913	0.879	0.913	0.913
ucr	NA	NA	0.918	1	1	0.957	0.967	0.871	0.967	0.967
ucrC	NA	NA	NA	0.918	0.918	0.896	0.893	0.935	0.892	0.893
ucrss	NA	NA	NA	NA	1	0.957	0.967	0.871	0.967	0.967
ucrss_wfilter	NA	NA	NA	NA	NA	0.957	0.967	0.871	0.967	0.967
uc_fast	NA	NA	NA	NA	NA	NA	0.912	0.876	0.912	0.912

ucr_fast	NA	NA	NA	NA	NA	NA	NA	0.855	1	1
ucrC_fast	NA	NA	NA	NA	NA	NA	NA	NA	0.854	0.855
ucrss_fast	NA	NA	NA	NA	NA	NA	NA	NA	NA	1
ucrss_fast_wfi lter	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Runtime comparison

Comparison of runtimes (as seconds of wall time) for each method on each data set.

	88-soil	moving-picture	whole-body
uc	1220	27748	1095
ucr	1358	46576	1082
ucrC	226	28572	388
ucrss	1493	47207	1212
ucrss_wfilter	1885	76061	2088
uc_fast	914	23510	489
ucr_fast	1052	19371	621
ucrC_fast	44	2428	68
ucrss_fast	1021	23710	707
ucrss_fast_wfilter	1525	52811	1661

Runtime comparisons (subsampled open-reference OTU picking variants)

Comparison of runtimes (as seconds of wall time) for subsampled and "classic" open-reference OTU picking methods with variations on the default parameters.

abbreviation	moving-picture
ucr_fast_O29_r82	21737
ucr_fast_O29_r97	16241
ucrss_fast_O29_r82	17812
ucrss_fast_O29_r97	16169
ucrss_fast_O29_s1	14911

OTU counts by environment

Comparison of OTUs with closed-reference and open-reference OTU picking by biome in the Earth Microbiome Project dataset.

	Average de novo OTUs (10K sequences per sample)	SD de novo OTUs (10K sequences per sample)	Average Reference OTUs (10k sequences per sample)	SD Reference OTUs (10k sequences per sample)	% novel diversity (10k seqs per sample)	% error novel diversity (10K seqs per sample)	number of samples
EnvironmentalBiome							
mangrove biome	2169	1159	354	73	0.86	0.46	7
tropical humid forests	2398	260	397	35	0.858	0.094	26
tundra biome	1771	403	312	117	0.85	0.201	110
deserts and xeric shrubland biome	3917	127	707	15	0.847	0.028	7
taiga	2598	102	505	35	0.837	0.035	4
marine biome	2040	1048	484	410	0.808	0.446	890
aquatic biome	714	299	177	199	0.801	0.403	762
freshwater biome	768	541	194	120	0.798	0.576	375
warm deserts and semideserts	2386	473	607	147	0.797	0.166	97
tropical and subtropical moist broadleaf forest biome	3072	125	846	18	0.784	0.032	2
temperate needle-leaf forests or woodlands	2836	159	785	132	0.783	0.057	21
polar biome	1721	886	483	218	0.781	0.414	277
tropical and subtropical coniferous forest biome	1993	256	579	94	0.775	0.106	3
mixed island systems	1552	618	511	203	0.752	0.315	124
marginal sea	1795	325	611	225	0.746	0.164	7
temperate coniferous forest biome	2504	1206	885	201	0.739	0.361	19
mediterranean forests, woodlands, and shrub biome	695	361	275	195	0.717	0.424	371
large river biome	1844	629	743	369	0.713	0.282	5
terrestrial biome	2714	222	1138	163	0.705	0.072	627
nest of bird	821	276	355	138	0.698	0.262	313
Temperate broadleaf and mixed forest biome	1910	491	879	235	0.685	0.195	14
temperate	2745	290	1315	164	0.676	0.082	696

grasslands							
animal-associated habitat	758	329	376	240	0.668	0.359	1036
mammalia-associated habitat	973	357	583	222	0.625	0.27	1918
Cold-winter (continental) deserts and semideserts	847	210	551	215	0.606	0.215	102
Temperate grasslands, savannas, and shrubland biome	1688	272	1497	275	0.53	0.121	85
human-associated habitat	292	242	590	366	0.331	0.498	1597

Significantly different OTUs by environmental metadata

Top 10 OTUs identified as significantly different across (a) binned pH in 88-soils, (b) body site in moving-pictures, and (c) body site in whole-body.

(a)

OTU	taxonomy	Test-Statistic
113212	k__Bacteria;p__Acidobacteria;c__DA052;o__Ellin6513;f__;g__;s__	55.859
1123837	k__Bacteria;p__Actinobacteria;c__Rubrobacteria;o__Rubrobacterales;f__Rubrobacteraceae;g__Rubrobacter;s__	50.433
New.ReferenceOTU22	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__;g__;s__	49.172
252012	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Sinobacteraceae;g__;s__	48.65
843189	k__Bacteria;p__Acidobacteria;c__Solibacteres;o__Solibacterales;f__Solibacteraceae;g__Candidatus Solibacter;s__	47.006
1127423	k__Bacteria;p__Acidobacteria;c__Acidobacteriia;o__Acidobacteriales;f__Koribacteraceae;g__;s__	43.87
1129210	k__Bacteria;p__Acidobacteria;c__Acidobacteriia;o__Acidobacteriales;f__Koribacteraceae;g__;s__	43.804
831520	k__Bacteria;p__Actinobacteria;c__Rubrobacteria;o__Rubrobacterales;f__Rubrobacteraceae;g__Rubrobacter;s__	43.625
1139779	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria	41.863
804187	k__Bacteria;p__Acidobacteria;c__[Chloracidobacteria];o__RB41;f__;g__;s__	41.151

(b)

OTU	taxonomy	Test-Statistic
368134	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus;s__epidermidis	1599.696
3154070	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__uniformis	1625.703
1000986	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae;g__Corynebacterium;s__	1630.009
1992	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__	1728.164
4304475	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__	1545.445
191238	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus;s__	1546.436
187665	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__;s__	1474.529
4396297	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__;s__	1585.015
3903651	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira;s__	1670.188
3472078	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__	1783.488

(c)

OTU	taxonomy	Test-Statistic
4326219	k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Campylobacteraceae;g__Campylobacter;s__	363.881
New.CleanUp.ReferenceOTU222	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella;s__melaninogenica	358.02
4325533	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__;s__	349.852
New.CleanUp.ReferenceOTU17550	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Veillonella;s__parvula	337.656
316732	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Lachnospira;s__	337.309
4346374	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__uniformis	331.433
4458959	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Veillonella	329.772

3866487	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Oribacterium;s__	323.488
4391641	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;g__Haemophilus;s__parainfluenzae	312
175751	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__;s__	305.531