De novo clustering methods out-perform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units

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Abstract

Background. 16S rRNA gene sequences are routinely assigned to operational taxonomic units (OTUs) that are then used to analyze complex microbial communities. A number of methods have been employed to carry out the assignment of 16S rRNA gene sequences to OTUs leading to confusion over which method is the most rigorous. A recent study suggested that a clustering method should be selected based on its ability to generate stable OTU assignments that do not change as additional sequences are added to the dataset. In contrast, we contend that the ability of the method to properly represent the distances between the sequences is more important.

Methods. Our analysis implemented five *de novo* clustering algorithms including the single linkage, complete linkage, average linkage, abundance-based greedy clustering, distance-based greedy clustering, and the open and closed-reference methods. Using two previously published datasets we used the Matthew's Correlation Coefficient (MCC) to assess the stability and quality of OTU assignments.

Results. The stability of OTU assignments did not reflect the quality of the assignments. Depending on the dataset being analyzed, the average linkage and the distance and abundance-based greedy clustering methods generated more robust OTUs than the open and closed-reference methods. We also demonstrated that for the greedy algorithms VSEARCH produced assignments that were comparable to those produced by USEARCH making VSEARCH a viable free and open source alternative to USEARCH. Further interrogation of the reference-based methods indicated that when USEARCH is used to identify the closest reference, the OTU assignments were sensitive to the order of the reference sequences because the reference sequences can be identical over the region being considered. More troubling was the observation that while both USEARCH and VSEARCH have a high level of sensitivity to detect reference sequences, the specificity of those matches was poor relative to the true best match.

Discussion. Our analysis calls into question the quality and stability of OTU assignments generated by the open and closed-reference methods as implemented in current version of QIIME. This study demonstrates that *de novo* methods are the most rigorous and that the quality of clustering

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assignments needs to be assessed for multiple methods to identify the optimal clustering method for a particular dataset.

Introduction

The ability to affordably generate millions of 16S rRNA gene sequences has allowed microbial ecologists to thoroughly characterize the microbial community composition of hundreds of samples. To simplify the complexity of these large datasets, it is helpful to cluster sequences into meaningful bins. These bins, commonly known as operational taxonomic units (OTUs), are used to compare the biodiversity contained within and between different samples (Schloss & Westcott, 2011). Such comparisons have enabled researchers to characterize the microbiota associated with the human body (e.g. Huttenhower et al., 2012), soil (e.g. Shade et al., 2013), aquatic ecosystems (e.g. Gilbert et al., 2011), and numerous other environments. Within the field of microbial ecology, a convention has emerged where sequences are clustered into OTUs using a threshold of 97% similarity or a distance of 3%. One advantage of the OTU-based approach is that the definition of the bins is operational and can be changed to suit the needs of the particular project. However, with the dissemination of clustering methods within software such as mothur (Schloss et al., 2009), QIIME (Caporaso et al., 2010), and other tools (Edgar, 2010, Sun et al. (2009), Mahé et al. (2014), Edgar (2013), Cai & Sun (2011)), it is important to understand how different clustering methods implement this conventional OTU threshold. Furthermore, it is necessary to understand how the selected method affects the precision and accuracy of assigning sequences to OTUs. Broadly speaking, three approaches have been developed to assign sequences to OTUs.

The first approach has been referred to as phylotyping (Schloss & Westcott, 2011) or closed reference clustering (Navas-Molina et al., 2013). This approach involves comparing sequences to a curated database and then clustering sequences into the same OTU that are similar to the same reference sequence. Reference-based clustering methods suffer when the reference does not adequately reflect the biodiversity of the community. If a large fraction of sequences are novel, then they cannot be assigned to an OTU. In addition, the reference sequences are selected because they are less than 97% similar to each other over the full length of the gene; however, it is known that the commonly used variable regions within the 16S rRNA gene do not evolve at the same rate as the full-length gene (Schloss & Westcott, 2011). Thus, a sequence representing a fragment of the gene may be more than 97% similar to multiple reference sequences. Therefore, defining OTUs in the closed-reference approach is problematic because two sequences might be 97% similar to the same reference sequence, but they may only be 94% similar to each other. Alternatively, a seuquence may be equally similar to two or more reference sequences. A subtle alternative to this approach is to use a classifier to assign a taxonomy to each sequence so that sequences can be clustered at a desired level within the Linnean taxonomic hierarchy (Schloss & Westcott, 2011). The strength of the reference based approach is that the methods are generally fast, scaling linearly with the number of sequences being clustered.

The second approach has been referred to as distance-based (Schloss & Westcott, 2011) or *de novo* clustering (Navas-Molina et al., 2013). In this approach, the distance between sequences is used to cluster sequences into OTUs rather than the distance to a reference database. In contrast to the efficiency of closed-reference clustering, the speed of hierarchical *de novo* clustering methods scale quadratically with the number of unique sequences. The expansion in sequencing throughput combined with sequencing errors inflates the number of unique sequences resulting in the need for large amounts of memory and time to cluster the sequences. If error rates can be reduced through stringent quality control measures, then these problems can be overcome (Kozich et al., 2013). As an alternative, heuristics have been developed to approximate the clustering of hierarchical methods (Edgar, 2010, Sun et al. (2009), Mahé et al. (2014)). One critique of *de novo* approaches is that OTU assignments are sensitive to the input order of the sequences (He et al., 2015, Mahé et al. (2014)). Whether the differences in assignments is meaningful is unclear; however, the variation in results could represent equally valid clustering of the data. The strength of *de novo* clustering is its independence of references for carrying out the clustering step. After clustering, the classification of each sequence can be used to obtain a consensus classification for the OTU (Schloss & Westcott, 2011). For this reason, *de novo* clustering has been preferred across the field.

The third approach, open-reference clustering, is a hybrid of the closed-reference and *de novo* approaches (Navas-Molina et al., 2013; Rideout et al., 2014). Open-reference clustering involves performing closed-reference clustering followed by *de novo* clustering on those sequences that are not sufficiently similar to the reference. In theory, this method should exploit the strengths of both closed-reference and *de novo* clustering; however, the different OTU definitions employed by closed-reference and *de novo* clustering pose a possible problem when the methods are combined.

An alternative to this approach has been to classify sequences to a bacterial family or genus and then assigned to OTUs within those levels using the average linkage method (Schloss & Westcott, 2011). For example, all sequences assigned to the *Porphyromonadaceae* would then be assigned to OTUs using the average linkage method using a 3% distance threshold. Those sequences that did not classify to a known family would also be clustered using the average linkage method. An advantage of this approach is that it lends itself nicely to parallelization since each taxonomic group is seen as being independent and can be processed separately. Such an approach would overcome the difficulty of mixing OTU definitions between the closed-reference and *de novo* approaches.

The growth in options for assigning sequences using each of these three broad approaches has been considerable. It has been difficult to objectively assess the quality of OTU assignments. Some have focused on the time and memory required to process a dataset (Sun et al., 2009; Cai & Sun, 2011; Rideout et al., 2014, Mahé et al. (2014)). These are valid parameters to assess when judging a clustering method, but have little to say about the quality of the OTU assignments. Others have attempted to judge the quality of a method by its ability to generate data that parallels classification data (Sun et al., 2011; Cai & Sun, 2011; Chen et al., 2013; Edgar, 2013). This approach is problematic because bacterial taxonomy often reflects historical biases amongst bacterial systematicists. Furthermore, it is well known that the rates of evolution across lineages are not the same (Wang et al., 2007; Schloss, 2010). Others have assessed the quality of clustering based on their ability to generate the same OTUs generated by other methods (Rideout et al., 2014; Schmidt, Rodrigues & Mering, 2014). This is problematic because it does not solve the fundamental question of which method is most correct. We recently proposed an approach for evaluating OTU assignments using the distances between pairs of sequences (Schloss & Westcott, 2011). Those sequences that were similar to each other and found in the same OTU were called true positives while those that were similar and found in different OTUs were called false negatives. Meanwhile, those sequences that were different from each other and found in the same OTU were called false positives and those that were dissimilar and found in different OTUs were called true negatives. Counting the frequency of these different classes allowed us to judge how each method balanced the ratio of true positives and negatives to false positives and negatives using the Matthew's correlation coefficient (MCC; Matthews, 1975). This is an objective approach to assessing the quality of the OTU assignments.

A recent analysis by He and colleagues (2015) attempted to characterize the three general clustering approaches by focusing on what they called stability. They defined stability as the ability of a method to provide the same clustering on a subset of the data as was found in the full dataset. Their concept of stability did not account for the accuracy of the OTU assignments and instead focused on the precision of the assignments. A method may be very precise, but low in accuracy. In the current analysis, we assessed the accuracy and precision of the various clustering methods. Building on our previous analysis of clustering methods, our hypothesis was that the methods praised by the He study for their stability actually suffered a lack of accuracy. In addition, we assess these parameters in light of sequence quality using the original 454 dataset and a larger and more modern dataset generated using the MiSeq platform.

Results and Discussion

Summary and replication of He study. We obtained the Canadian soil dataset from Roesch et al. (2007) and processed the sequences as described by He and colleagues. Using these data, we reconsidered three of the more critical analyses performed in the He study.

First, we sought to quantify whether the OTU assignments observed for a subset of the data represented the same assignments that were found with the full dataset. The He study found that when they used the open and closed-reference methods the OTUs formed using the subsetted data most closely resembled those of the full dataset. Among the *de novo* methods they observed that the abundance-based greedy clustering (AGC) method generated the most stable OTUs followed by the single linkage (SL), distance-based greedy clustering (DGC), complete linkage (CL), and average linkage (AL) methods. We first sought to assess the calculated the MCC for the OTU assignments generated by each of the clustering methods using 20, 40, 60, and 80% of the sequences relative to the OTU composition formed by the methods using the full dataset (Figure 1A). Similar to the He study, we replicated each method and subsampled to the desired fraction of

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the dataset 30 times. Multiple subsamplings was necessary because a random number generator is used in some of the methods to break ties where pairs of sequences have the same distance between them. Across these sequencing depths, we observed that the stability of the OTUs generated by the SL and CL methods were highly sensitive to sampling effort relative to the OTUs generated by the AL, AGC, and DGC methods (Figure 1A). Our results (Figure 1B) largely confirmed those of Figure 4C in the He study with one notable exception. The He study observed a broad range of MCC values among their AL replicates when analyzing OTUs generated using 60% of the data. This result appeared out of character and was not explained by the authors. They observed a mean MCC value of approximately 0.63 (95% confidence interval between approximately 0.15 and 0.75). In contrast, we observed a mean value of 0.93 (95% confidence interval between 0.91 and 0.95). This result indicates that the AL assignments were far more stable than indicated in the He study. Regardless, although the assignments are quite stable, it does support the assertion that the OTU assignments observed for the subset of the data do not perfectly match the assignments that were found with the full dataset as they did with the reference-based methods; however, the significance of these differences is unclear.

Second, the He study and the original Roesch study showed that rarefaction curves calculated using CL-generated OTU assignments obtained using a portion of the dataset did not overlap with rarefaction curves generated using OTU assignments generated from the full dataset. The He and Roesch studies both found that the CL method produced fewer OTUs in the subset than in the rarefied data. In addition, the He study found that the SL method produced more OTUs, the AGC produced fewer, and the other methods produced similar numbers of OTUs than expected when comparing the subsetted data to the rarefied data. Our results support those of these previous studies (Figure 2). It was clear that inter-method differences were generally more pronounced than the differences observed between rarefying from the full dataset and from clustering the subsetted data. The number of OTUs observed was largest using the CL method, followed by the open-reference method. The AL, AGC, and DGC methods all provided comparable numbers of OTUs. Finally, the closed-reference and SL methods generated the fewest number of OTUs.

Third, the authors attempted to describe the effects of the OTU assignment instability on comparisons of communities. They used Adonis to test whether the community structure represented in subsetted communities resembled that of the full dataset when only using the unstable OTUs (Anderson, 2001). Although they were able to detect significant p-values, they appeared to be of marginal biological significance. Adonis R statistics close to zero indicate the community structures from the full and subsetted datasets overlapped while values of one indicate the communities are completely different. The He study observed adonis R statistics of 0.02 (closed-reference), 0.03 (open-reference), 0.07 (CL, AGC, DGC), and 0.16 (SL and AL). Regardless of the statistical or biological significance of these results, the analysis does not make sense since, by definition, representing communities based on their unstable OTUs would yield differences. Furthermore, the *de novo* and open-reference approaches do not consistently label the OTUs that sequences belong to when the clustering methods are run multiple times with different random number seeds. To overcome this, the authors selected representative sequences from each OTU and used those representative sequences to link OTU assignments between the different sized sequence sets. The justification for this analysis is specious as the OTU assignments are based on the data available in the dataset when the sequences are clustered and comparing assignments in this manner are irreconcilable. It is not surprising that the only analysis that did not provide a significant p-value was for the closed-reference analysis, which is the only analysis that provides consistent OTU labels. Finally, the authors built off of this analysis to count the number of OTUs that were differentially represented between the subsetted and full datasets by each method. This analysis assumes that the OTUs generated using the full dataset were correct, which was an unsubstantiated assumption since the authors did not assess the quality of the OTU assignments. Because this analysis was so poorly designed, we did not seek to reproduce it.

OTU assignment methods vary in their accuracy. More important than the stability of OTUs is whether sequences are assigned to the correct OTUs. A method can generate highly stable OTUs, but the OTU assignments may be meaningless if they poorly represent the specified cutoff and the actual distance between the sequences. To assess the quality of OTU assignments by the various methods, we made use of the pairwise distance between the unique sequences to count the number of true positives and negatives and the number of false positives and negatives for each method and sampling depth. This enabled us to calculate the average MCC value as a measure of a method's accuracy and its variation as a measure of its precision. We made three important observations. First, each of the *de novo* methods varied in how sensitive their MCC values were to additional sequences (Figure 1C). The SL and CL methods were the most sensitive; however, the accuracy of the OTU assignments did not meaningfully differ when 80 or 100% of the data were assigned to OTUs using the *de novo* methods. Second, the AL method had higher MCC values than the other methods followed by DGC, AGC, CL, open-reference, and closed-reference, and SL (Figure 1D). Third, with the possible exception of the CL method, the MCC values for each of the methods only demonstrated a small amount of variation between runs of the method with a different ordering of the input sequences. This indicates that although there may be variation between executions of the same method, they produce OTU assignments that are equally good. Revisiting the concept of stability, we question the value of obtaining stable OTUs when the full dataset is not optimally assigned to OTUs. Our analysis indicates that the most rigorous method for assigning the Canadian soils sequences to OTUs using a 97% threshold was the AL method.

Deep sampling of 16S rRNA genes. Three factors make the Canadian soil dataset less than desirable to evaluate clustering methods. First, it was one of the earliest 16S rRNA gene sequence datasets published using the 454 FLX platform. Developments in sequencing technology now permit the sequencing of millions of sequences for a study. In addition, because the original Phred quality scores and flowgram data are not available, it was not possible for us to adequately remove sequencing errors (Schloss, Gevers & Westcott, 2011). The large number of sequences that one would expect to remain in the dataset are likely to negatively affect the performance of all of the clustering methods. Second, the dataset used in the He study covered the V9 region of the 16S rRNA gene. For a variety of reasons, this region is not well represented in databases, including the reference database used by the closed and open-reference methods. Of the 99,322 sequences in the default QIIME database, only 48,824 fully cover the V9 region. In contrast, 99,310 of the sequences fully covered the V4 region. Inadequate coverage of the V9 region would adversely affect the ability of the reference-based methods to assign sequences to OTUs. Third, our previous analysis has shown that the V9 region evolves at a rate much slower than the rest of the gene (Schloss, 2010). With these points in mind, we compared the clustering assignment for each of these methods using a time series experiment that was obtained using mouse feces (Schloss et al., 2012; Kozich et al., 2013). The MiSeq platform was used to generate 2,825,000 sequences from the V4 region of the 16S rRNA gene of 360 samples. Parallel sequencing of a mock community indicated that the sequencing error rate was approximately 0.02% (Kozich et al., 2013). Although no dataset is perfect for exhaustively testing these clustering methods, this dataset was useful for demonstrating several points. First, when using 60% of the data, the stability relationships amongst the different methods were similar to what we observed using the Canadian soil dataset (Figure 3AB). With the exception for the clusters generated using CL, the methods all performed very well with stabilities greater than 0.91. Second, the MCC values calculated relative to the distances between sequences were generally higher than was observed for the Canadian soil dataset for all of the methods except the CL and SL methods. Surprisingly, the MCC values for the DGC (0.77) and AGC (0.76) methods were comparable to the AL method (0.76; Figure 3CD). This result suggests that the optimal method is likely to be database-dependent.

Finally, as was observed with the Canadian soil dataset, there was little variation in MCC values observed among the 30 randomizations. Therefore, although the methods have a stochastic component, the OTU assignments do not vary meaningfully between runs. The results from both the Canadian soil and murine microbiota datasets demonstrate that the *de novo* methods can generate stable OTU assignments and that the assignments are highly reproducible. Most importantly, these analyses demonstrate that the OTU assignments using the AL, AGC, and DGC *de novo* methods are consistently more robust than either of the reference-based methods.

Evalution of Swarm as an alternative de novo clustering algorithm. The Swarm algorithm is a recently proposed *de novo* method for assigning sequences to OTUs that identifies clusters of sequences based on the number of differences between each other without applying a distance threshold (Mahé et al., 2014). Swarm was originally validated by comparing the results against the expected clusters formed based on the taxonomic composition of a mock community. Similar to the authors of the He study, the Swarm developers suggest that method are needed that are insensitive to input order. Use of Swarm on the Canadian soil and murine datasets demonstrated that similar to the other *de novo* methods, Swarm's OTU assignments changed as sequences were added (Figures 1A and 3A). When we compared the OTU assignments for both datasets when using all of the sequence data, the variation in MCC values across the 30 randomizations were not meaningfully different (Figures 1D and 3D). Most importantly, when we selected the distance threshold that optimized the MCC value, the quality of the OTU assignments was close to that of the AL assignments when using the Canadian soil dataset and considerably worse than that of the murine dataset (Figures 1D and 3D). Interestingly, the distance thresholds that resulted in the largest MCC values were 3 and 2% for the Canadian soil and murine datasets, respectively. This suggests that OTU definitions are not consistent across datasets when using the Swarm algorithm. Finally, the Swarm developers indicated that hierarchical *de novo* algorithms were too impractical to use on large MiSeq-generated datasets. Our ability to apply AL to the large mouse dataset and even larger datasets suggests (e.g. Schubert, Sinani & Schloss, 2015, Zackular et al. (2015)) that it is not necessary to sacrifice OTU assignment quality for speed.

Evalution of an open-source alternative to USEARCH. For some datasets the AGC and DGC methods appear to perform as well or better than the hierarchical clustering methods. As originally described in the He study, the AGC and DGC methods utilized the USEARCH program and the DGC method is used for clustering in UPARSE (Edgar, 2010, Edgar (2013)). The source code for USEARCH is not publicly available and only the 32-bit executables are available for free to academic users. Access for non-academic users and those needing the 64-bit version is available commercially from the developer. An alternative to USEARCH is VSEARCH, which is being developed in parallel to USEARCH as an open-source alternative. One subtle difference between the two programs is that USEARCH employs a heuristic to generate candidate alignments whereas VSEARCH generates the actual global alignments. The VSEARCH developers claim that this difference enhances the sensitivity of VSEARCH relative to USEARCH. Using the two datasets, we determined whether the AGC and DGC methods, as implemented by the two programs, yielded OTU assignments of similar quality. In general the overall trends that we observed with the USEARCH-version of AGC and DGC were also observed with the VSEARCH-version of the methods (Figure 4). When we compared the two implementations of the AGC and DGC methods, the OTUs generated by the VSEARCH-version of the methods were as stable or more stable than the USEARCH-version when using 60% of the datasets. In addition, the MCC values for the entire datasets, calculated relative to the distance matrix, were virtually indistinguishable. These results are a strong indication that VSEARCH is a suitable and possibly better option for executing the AGC and DGC methods.

Problems with reference-based clustering in general and as implemented in QIIME. The He study and our replication attempt validated that the closed-reference method generated perfectly stable OTUs. This was unsurprising since, by definition, the method is designed to return one-to-one mapping of reads to a reference. Furthermore, because it treats the input sequences independently the input order or use of a random number generator is not an issue. An important test that was not performed in the He study was to determine whether the clustering was sensitive to the order of the sequences in the database. The default database used in QIIME, which was also used in the He study, contains full-length sequences that are at most 97% similar to each other. We randomized the order of the reference sequences 30 times and used them to carry out the closed-reference method with the full murine dataset, which contained 32,106 unique sequences. Surprisingly, we observed that the number of OTUs generated was not the same in each of the randomizations. On average there were 28,059 sequences that mapped to a reference OTU per randomization (range from 28,007 to 28,111). The original ordering of the reference resulted in 27,876 sequences being mapped, less than the minimum observed number of mapped sequences when the references were randomized. This surprising result was likely due to the performance of the USEARCH heuristic. To test this further, we substituted VSEARCH for USEARCH in the closed-reference method. When we used VSEARCH the original ordering of the reference sequences and all randomizations were able to map 27,737 sequences to reference OTUs. When we calculated the true distance between each of the murine sequences and the references, we were able to map 28,238 of the murine sequences to the reference sequences when using a 97% similarity threshold without the use of a heuristic. This result indicates that the closed reference approach, whether using USEARCH or VSEARCH, does not exhaustively or accurately map reads to the closest reference. To quantify this further, we calculated the MCC for the USEARCH and VSEARCH assignments relative to the assignments using the non-heuristic approach. Using USEARCH the average MCC was 0.78 (range: 0.75 to 0.80) and using VSEARCH the average MCC was 0.65 (range: 0.64 to 0.66). The two methods had similar sensitivities (USEARCH: 0.98 and VSEARCH: 0.97), but the USEARCH specificity (0.73) was considerably higher than VSEARCH (0.60). Overall, these results indicate that although heuristic approaches may be fast, they do a poor job of mapping reads to the correct reference sequence relative to non-heuristic approaches.

We also observed that regardless of whether we used USEARCH or VSEARCH, the reference OTU labels that were assigned to each OTU differed between randomizations. When we used USEARCH to perform closed-reference clustering, an average of 57.38% of the labels were shared between pairs of the 30 randomizations (range=56.14 to 59.55). If we instead used VSEARCH an average of 56.23% of the labels were shared between pairs of the 30 randomizations (range=53.48 to 59.12). To better understand this result, we further analyzed QIIME's reference database. We hypothesized that within a given region there would be sequences that were more than 97% similar and possibly identical to each other. When a sequence was used to search the randomized databases, it would encounter a different reference sequence as the first match with each randomization. Among those reference sequences that fully overlap the V4 region, there were 7,785 pairs of sequences that were more than 97% similar to each other over the full length of the 16S rRNA gene. When the extracted V4 sequences were dereplicated, we identified 88,347 unique sequences. Among these dereplicated V4 sequences there were 311,430 pairs of sequences that were more than 97% similar to each other. The presence of duplicate V4 reference sequences explains the lack of labeling stability when using either USEARCH or VSEARCH to carry out the closed-reference method. We suspect that the reference database was designed to only include sequences that were at most 97% similar to each other as a way to overcome the limitations of the USEARCH search heuristic.

Beyond comparing the abundance of specific OTUs across samples, the reference database is used in the open and closed-reference methods to generate OTU labels that are used in several downstream applications. It is commonly used to extract information from a reference phylogenetic tree to carrying out UniFrac-based analyses (Hamady, Lozupone & Knight, 2009) and to identify reference genomes for performing analyses such as PICRUSt (Langille et al., 2013). Because these downstream applications depend on the correct and unique labeling of the OTUs, the lack of stability of the labeling is problematic. As one illustration of the effects that incorrect labels would have on an analysis, we asked whether the duplicate sequences had the same taxonomies. Among the 3,132 reference sequences that had one duplicate, 443 had discordant taxonomies. Furthermore, among those 1,699 sequences with two or more duplicates, 698 had discordant taxonomies. Two sequences mapped to 30 and 10 duplicate sequences and both contained 7

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different taxonomies. Among the sequences within the database, there was also a sequence that had 131 duplicates and contained 5 different taxonomies. When we analyzed the 28,238 sequences that mapped to the reference sequences using a non-heuristic approach, we observed that 18,315 of the sequences mapped to more than one reference sequence. Of these sequences, 13,378 (73.04%) mapped to references that were identical over the V4 region and 4,937 (26.96%) mapped equally well to two or more references that were not identical over the V4 region. Among the combined 18,315 sequences that mapped to multiple reference sequences, the taxonomy of the multiple reference sequences conflicted for 3,637 (19.86%). Together, these results demonstrate some of the considerable problems with the reference-based clustering of sequences.

Conclusions

It is worth noting that the entire design of the He study was artificial. First, their analysis was based on a single soil sample. Researchers generally have dozens or hundreds of samples that are pooled and clustered together to enable comparison across samples. Second, all of the sequence data from these datasets is pooled for a single analysis. It is unclear why anyone would ever perform an analysis based on a subset of their data. Because of these points, the value of identifying stable OTUs is unclear. Greater emphasis should be placed on obtaining an optimal balance between splitting similar sequences into separate OTUs and merging disparate sequences into the same OTU. Through the use of the pairwise distances between sequences, we were able to use the MCC to demonstrate that, in general, the AL method is consistently robust, but that Swarm, AGC, and DGC sometimes perform as well as AL. At least for the murine dataset, Swarm also could be among the least robust methods. Although there is concern that running the methods multiple times yields different clusterings, we have shown that there is little variation in their MCC values. This suggests that the different clusterings by the same method are equally good. Finally, it is impossible to obtain a clustering with no false positives or false negatives and the optimal method may vary by dataset. With this in mind, researchers are encouraged to calculate and report their MCC values for the AL method and at least one other method.

Our analysis of those methods that implemented USEARCH as a method for clustering sequences revealed that its heuristic limited its specificity. When we replaced USEARCH with VSEARCH, the clustering quality was as good or better. Although there may be parameters in USEARCH that can be tuned to improve the heuristic, these parameters are likely dataset dependent. Based on the data presented in this study, its availability as an open source, and free program, VSEARCH should replace USEARCH in the *de novo* clustering methods; however, USEARCH performed better than VSEARCH for closed-reference clustering. Furthermore, although not tested in our study, VSEARCH can be parallelized leading to potentially significant improvements in speed. Although USEARCH and VSEARCH do not utilize aligned sequences, it is important to note that a sequence curation pipeline including denoising, alignment, trimming to a consistent region of the 16S rRNA gene, and chimera checking are critical to making proper inferences (Schloss, Gevers & Westcott, 2011; Schloss, 2012; Kozich et al., 2013).

We have assessed the ability of reference-based clustering methods to capture the actual distance between the sequences in a dataset in parallel with *de novo* methods. Several studies have lauded both the open and closed-reference approaches for generating reproducible clusterings (Navas-Molina et al., 2013; Rideout et al., 2014; He et al., 2015), yet we have shown that both reference-based approaches did a poor job of representing the distance between the sequences compared to the *de novo* approaches. Although the OTU assignments are reproducible and stable across a range of library sizes, the reference-based OTU assignments are a poor representation of the data. We also observed that the assignments were not actually reproducible when the order of the reference sequences was randomized. When USEARCH was used, the actual number of sequences that mapped to the reference changed depended on the order of the reference. Perhaps most alarming was that the default order of the database provided the worst MCC of any of the randomizations we attempted. Even when we used VSEARCH to perform closed-reference clustering and were able to obtain a consistent clusterings, we observed that the labels on the OTUs differed between randomizations. Because the OTU labels are frequently used to identify representative sequences for those OTUs, variation in labels, often representing different taxonomic groups, will have a detrimental effect on the interpretation of downstream analyses.

Because the open-reference method is a hybrid of the closed-reference and DGC methods, it is also negatively affected by the various problems using USEARCH. An added problem with the open-reference method is that the two phases of the method employ different thresholds to define its OTUs. In the closed-reference step, sequences must be within a threshold of a reference to be in the same OTU. This means that two sequences that are 97% similar to a reference and are joined into the same OTU, may only be 94% similar to each other. In the DGC step, the goal is to approximate the AL method which requires that, on average, the sequences within an OTU are, on average, 97% similar to each other. The end result of the open-reference approach is that sequences that are similar to previously observed sequences are clustered with one threshold while those that are not similar to previously observed sequences are clustered with a different threshold.

As the throughput of sequencing technologies have improved, development of clustering algorithms must continue to keep pace. *De novo* clustering methods are considerably slower and more computationally intensive than reference-based methods and the greedy *de novo* methods are faster than the hierarchical methods. In our experience (Kozich et al., 2013), the most significant detriment to execution speed of the *de novo* methods has been the inadequate removal of sequencing error and chimeras. As the rate of sequencing error increases so do the number of unique sequences that must be clustered. The speed of the *de novo* methods scales approximately quadratically, so that doubling the number of sequences results in a four-fold increase in the time required to execute the method. The rapid expansion in sequencing throughput has been likened to the Red Queen in Lewis Carroll's, *Through the Looking-Glass* who must run in place to keep up to her changing surroundings (Schloss et al., 2009). Microbial ecologists must continue to refine clustering methods to better handle the size of the datasets, but they must also take steps to improve the quality of the underlying data. Ultimately, objective standards must be applied to assess the quality of the data and the quality of OTU clustering.

Methods

454 FLX-generated Roesch Canadian soil dataset. After obtaining the 16S rRNA gene fragments from GenBank (accessions EF308591-EF361836), we followed the methods outlined by the He study by removing any sequence that contained an ambiguous base, was identified as being a chimera, and fell outside a defined sequence length. Although they reported observing a total of 50,542 sequences that were represented by 13,293 unique sequences, we obtained a total of 50,946 sequences that were represented by 13,393 unique sequences. Similar to the He study, we randomly sampled, without replacement, 20, 40, 60, and 80% of the sequences from the full data set. The random sampling was repeated 30 times. The order of the sequences in the full dataset was randomly permuted without replacement to generate an additional 30 datasets. To perform the hierarchical clustering methods and to generate a distance matrix we followed the approach of the He study by calculating distances based on pairwise global alignments using the `pairwise.dist` command in mothur using the default Needleman-Wunsch alignment method and parameters. It should be noted that this approach has been strongly discouraged (Schloss, 2012). Execution of the hierarchical clustering methods was performed as described in the original He study using mothur (v.1.37) and using the QIIME (v.1.9.1) parameter profiles provided in the supplementary material from the He study for the greedy and reference-based clustering methods.

MiSeq-generated Murine gut microbiota dataset. The murine 16S rRNA gene sequence data generated from the V4 region using an Illumina MiSeq was obtained from http:/www.mothur.org/MiSeqDevelopmentData/StabilityNoMetaG.tar and was processed as outlined in the original study (Kozich et al., 2013). Briefly, 250-nt read pairs were assembled into contigs by aligning the reads and correcting discordant base calls by requiring one of the base calls to have a Phred quality score at least 6 points higher than the other. Sequences where it was not possible to resolve the disagreement were culled from the dataset. The sequences were then aligned to a SILVA reference alignment (Pruesse et al., 2007) and any reads that aligned outside of the V4 region were removed from the dataset. Sequences were pre-clustered by combining the abundances of sequences that differed by 2 or fewer nucleotides of a more abundant sequence. Each of the samples was then screened for chimeric sequences using the default parameters in UCHIME (Edgar et al., 2011). The resulting sequences were processed in the same manner as the Canadian soil dataset with the exception that the distance matrices were calculated based on the SILVA-based alignment.

Analysis of reference database. We utilized the 97% OTUs greengenes reference sequence and taxonomy data (v.13.8) that accompanies the QIIME installation. Because the greengenes reference alignment does a poor job of representing the secondary structure of the 16S rRNA gene (Schloss, 2010), we realigned the FASTA sequences to a SILVA reference alignment to identify the V4 region of the sequences.

Calculation of Matthew's Correlation Coefficient (MCC). The MCC was calculated by two approaches in this study using only the dereplicated sequence lists. First, we calculated the MCC to determine the stability of OTU assignments following the approach of the He study. We assumed that the clusters obtained from the 30 randomized full datasets were correct. We counted the number of sequence pairs that were in the same OTU for the subsetted dataset and the full dataset (i.e. true positives; TP), that were in different OTUs for the subsetted dataset and the full dataset (i.e. true negatives; TN), that were in the same OTU for the subsetted dataset and different OTUs in the full dataset (i.e. false positives; FP), and that were in different OTUs for the subsetted dataset and the same OTU in the full dataset (i.e. false negatives; FN). For each set of 30 random subsamplings of the dataset, we counted these parameters against the 30 randomizations of the full dataset. This gave 900 comparisons for each fraction of sequences being used in the analysis. The Matthew's correlation coefficient was then calculated as:

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

Second, we calculated the MCC to determine the quality of the clusterings as previously described (Schloss & Westcott, 2011). Briefly, we compared the OTU assignments for pairs of sequences to the distance matrix that was calculated between all pairs of aligned sequences. For each dataset that was clustered, those sequences that were in the same OTU and had a distance less than 3% were TPs, those that were in different OTUs and had a distance greater than 3% were TNs, those that were in the same OTU and had a distance greater than 3% were FPs, and those that were in different OTUs and had a distance less than 3% were FNs. The MCC was counted for each dataset using the formula above as implemented in the `sens.spec` command in mothur. To judge the quality of the Swarm-generated OTU assignments we calculated the MCC value using thresholds incremented by 1% between 0 and 5% and selected the threshold that provided the optimal MCC value.

Software availability. A reproducible workflow including all scripts and this manuscript as a literate programming document are available at https://github.com/SchlossLab/Schloss_Cluster_PeerJ_2015. The workflow utilized QIIME (v.1.9.1; Caporaso et al., 2010), mothur (v.1.37.0; Schloss et al., 2009), USEARCH (v.6.1; Edgar, 2010), VSEARCH (v.1.5.0; Rognes et al., 2015), Swarm (v.2.1.1; Mahé et al., 2014), and R (v.3.2.0; R Core Team, 2015). The SL, AL, and CL methods are called nearest neighbor (NN), average neighbor (AN), and furthest neighbor (FN) in mothur; we have used the terminology from the He study to minimize confusion. The knitr (v.1.10.5; Xie, 2013), Rcpp (v. 0.11.6; Eddelbuettel, 2013), rentrez (v. 1.0.0; Winter, Chamberlain & Guangchun, 2015), and jsonlite (v. 0.9.16; Ooms, 2014) packages were used within R.

Figures

Figure 1. Comparison of the stability (A, B) and quality (C, D) of *de novo* and reference-based clustering methods using the Canadian soil dataset. The average stability of the OTUs were determined by calculating the MCC with respect to the OTU assignments for the full dataset using varying sized subsamples (A). Thirty randomizations were performed for each fraction of the dataset and the average and 95% confidence interval are presented when using 60% of the data. The quality of the OTUs were determined by calculating the MCC with respect to the distances between the sequences using varying sized subsamples (C). Thirty randomizations were performed for each fraction of the dataset and the average and 95% confidence interval are presented when using the full dataset (D). The vertical gray line indicates in A and C indicates the fraction of the dataset represented in B and D, respectively. The optimum threshold for the Swarm-generated assignments was 3%.

Figure 2. The clustering methods varied in their ability to generate the same number of OTUs using a subset of the data as were observed when the full dataset was rarefied. The subsetted data are depicted by closed circles and the data from the rarefied full dataset is depicted by the open circles.

Figure 3. Comparison of the stability (A, B) and quality (C, D) of *de novo* and reference-based clustering methods using the murine dataset. The average stability of the OTUs were determined by calculating the MCC with respect to the OTU assignments for the full dataset using varying sized subsamples (A). Thirty randomizations were performed for each fraction of the dataset and the average and 95% confidence interval are presented when using 60% of the data. The quality of the OTUs were determined by calculating the MCC with respect to the distances between the sequences using varying sized subsamples (C). Thirty randomizations were performed for each fraction of the dataset and the average and 95% confidence interval are presented when using the full dataset (D). The vertical gray line indicates in A and C indicates the fraction of the dataset represented in B and D, respectively. The optimum threshold for the Swarm-generated assignments was 2%.

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Figure 4. The VSEARCH OTUs generated by the AGC and DGC methods were comparable to those generated using USEARCH.

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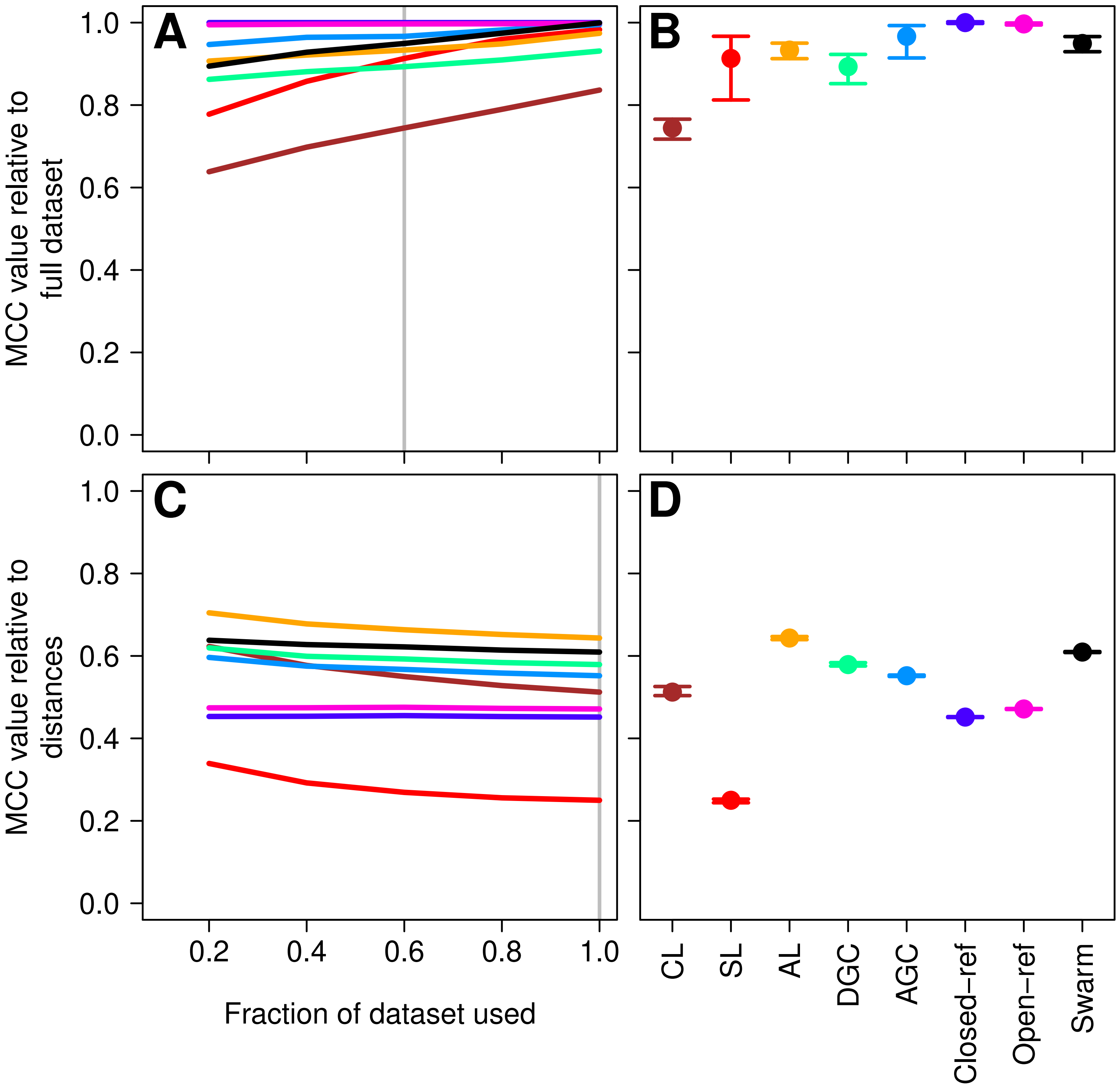
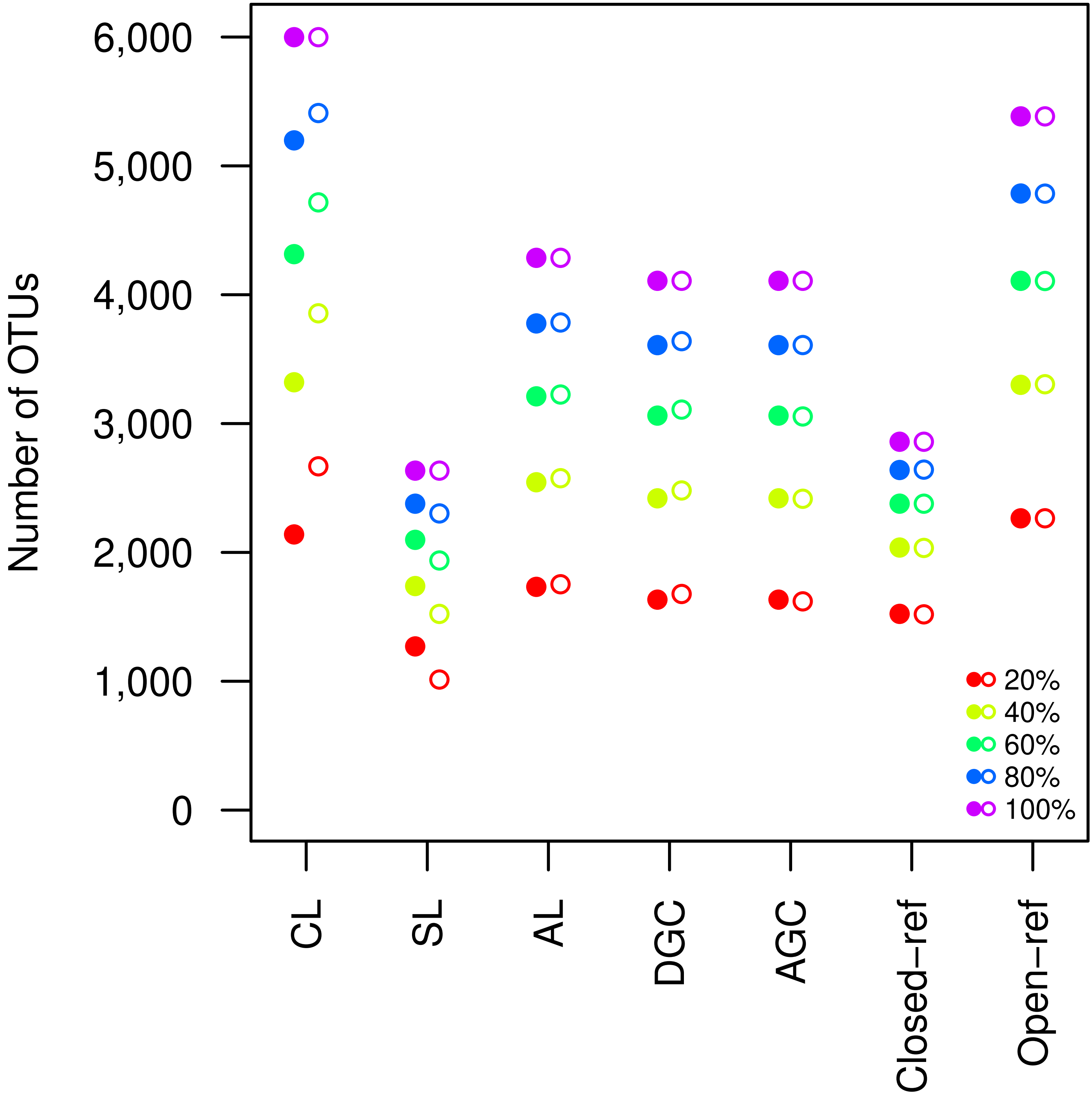

A
B
C
D
MCC value relative to full dataset
MCC value relative to distances
1.0
0.8
0.6
0.4
0.2
0.0
Fraction of dataset used
0.2
0.4
0.6
0.8
1.0
CL
SL
AL
DGC
AGC
Closed–ref
Open–ref
Swarm

519



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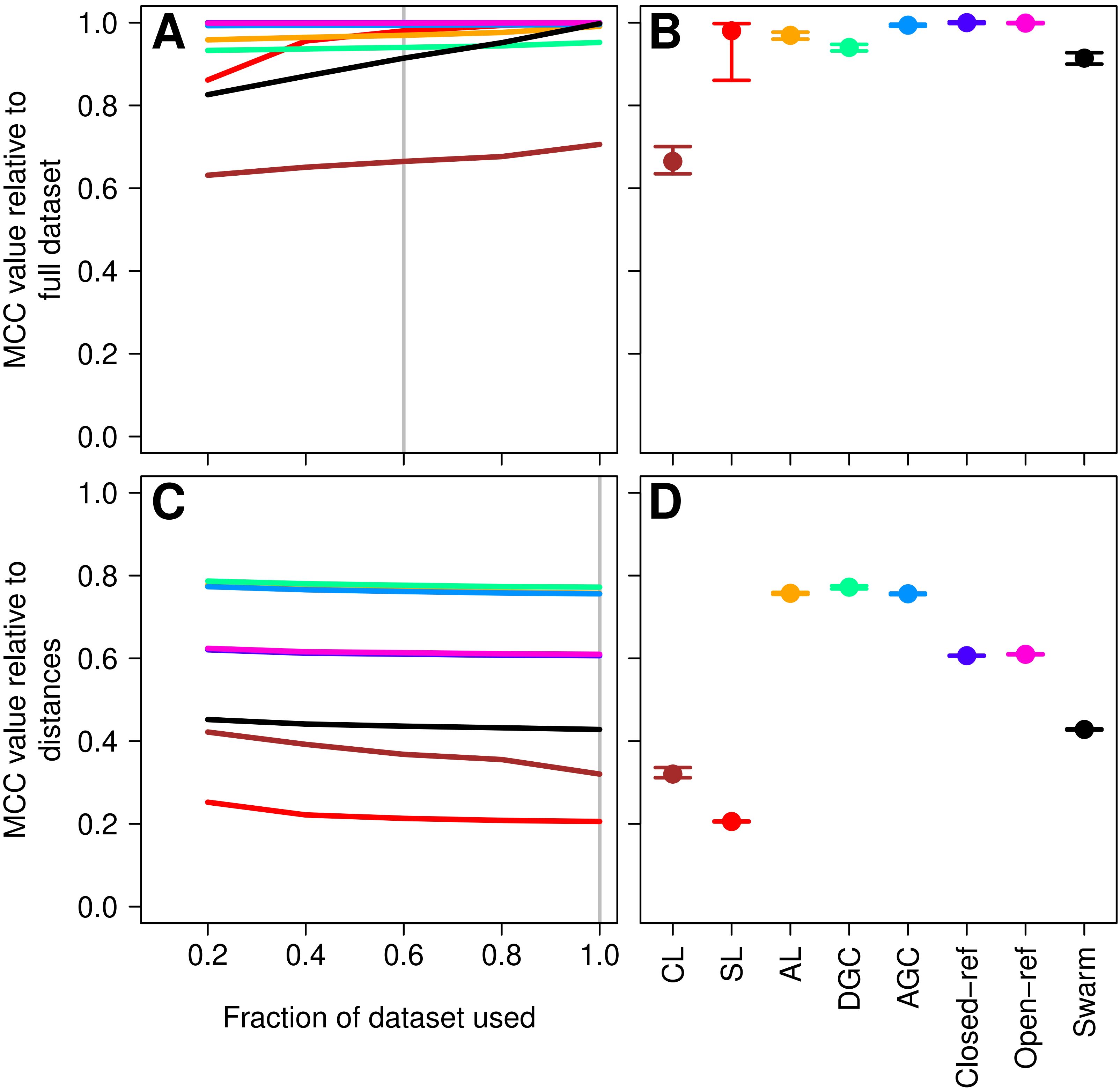
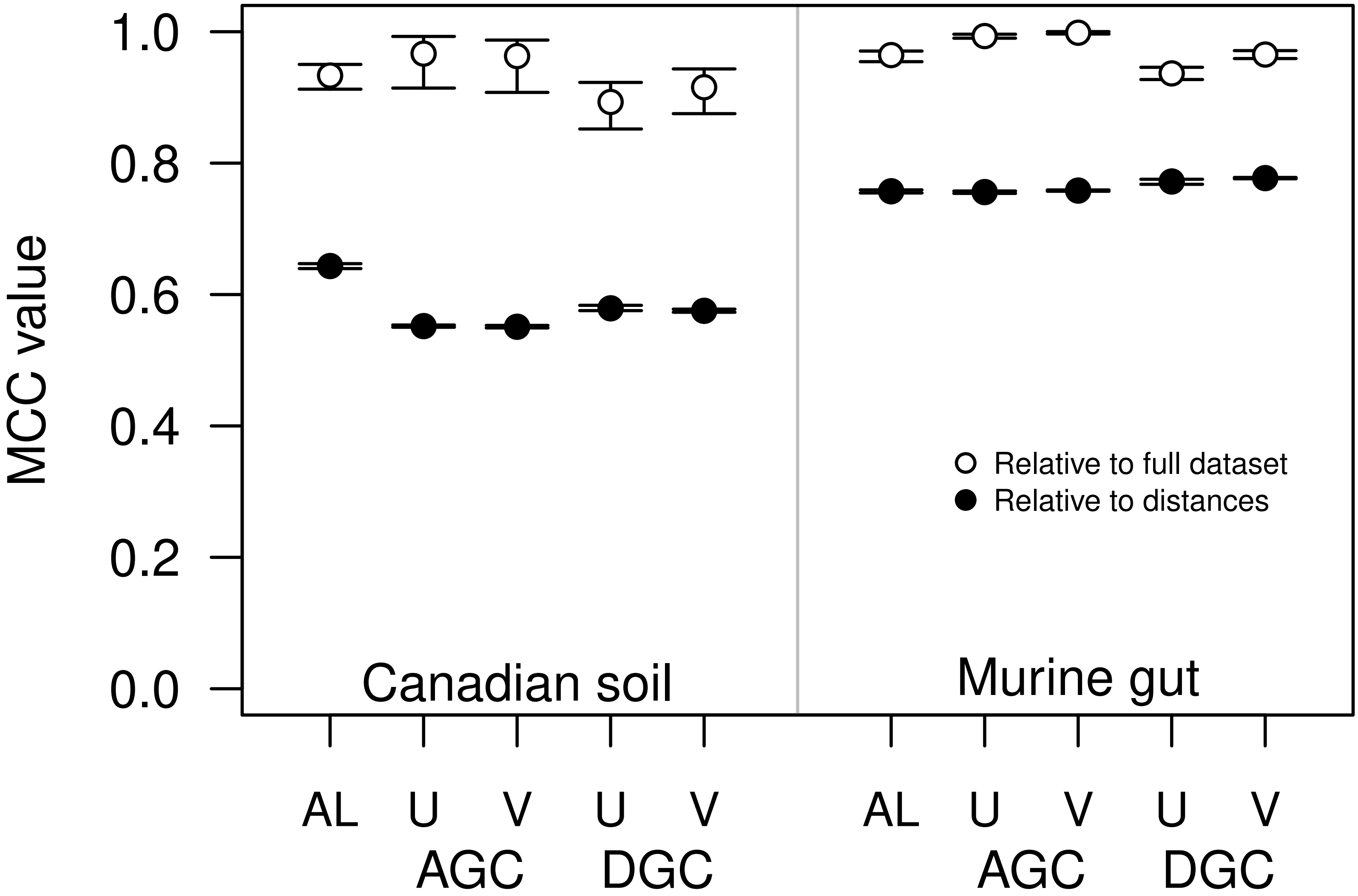
520


A
B
C
D
MCC value relative to full dataset
MCC value relative to distances
1.0
0.8
0.6
0.4
0.2
0.0
0.2
0.4
0.6
0.8
1.0
Fraction of dataset used
CL
SL
AL
DGC
AGC
Closed–ref
Open–ref
Swarm

521


1.0
0.8
0.6
0.4
0.2
0.0
MCC value
Canadian soil
Murine gut
○ Relative to full dataset
● Relative to distances
AL
U
V
U
V
AGC
DGC
AL
U
V
U
V
AGC
DGC

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