

Dear Dr. Eren,

Thank you for providing my co-author and I with a very fast review of our first submission. We were delighted by the generally positive and thorough comments provided by the two reviewers. Attached you will find a complete set of responses to each of their concerns. As you suggested, we streamlined our jargon and tempered our language. To the points raised by reviewer 2, we elected to use the MCC as was done in the original He paper for consistency. We have added text to the manuscript indicating that this probably wasn't the best choice of methods. Hopefully, the point is clear that this way of measuring stability is not ideal.

Please do not hesitate to contact us if you have additional comments or concerns.

Sincerely,

Sarah Westcott & Pat Schloss

Reviewer 1 (Anonymous)

Basic reporting

Code to reproduce the results has been provided in a github repository.

See general comments.

Experimental design

See general comments.

Validity of the findings

See general comments.

Comments for the author

This paper evaluates the “stability” and “quality” of several different OTU clustering methods. “Stability” is defined here as the consistency between OTU assignments made on subsets of the data and the full dataset. “Quality” is defined here as assigning sequencing reads that are <3% different to the same OTU, and sequencing reads >3% different to different OTUs. The authors broad conclusions are that while reference based clustering is more “stable”, that hierarchical de novo methods, particularly average linkage, produce OTUs of higher “quality”. The authors also make an important observation that the OTU assignments of commonly used reference methods depend on the ordering of the reference database.

This paper is largely a response to He et al. “Stability of operational taxonomic units: an important but neglected property for analyzing microbial diversity” which argued for reference based methods based on the “stability” of their OTU assignments.

This paper is well-written, makes several useful observations, and is clearly suitable for publication. It can be improved by certain clarifications. I recommend its acceptance with minor revisions, as detailed below.

More important points are preceded by an asterisk.

***Terminology:** Over the course of the paper, (at least) five different terms are used to refer to the goodness of a clustering: stability, rigor, robustness, quality and accuracy. Yet only two distinct concepts are explored (stability and quality/accuracy). I strongly recommend that the words “rigorous” and “robust” be struck from the manuscript entirely. They are never defined, are unclear, and the first impression they give does not correspond well to the actual results they are being used to describe.

We thank this Reviewer (and Reviewer 2) for expressing their concern that we are not sufficiently clear in our terminology. Part of the problem comes down to the multiple definitions of accuracy, precision, etc. in the classification and statistics literature. The multiple terms is symptomatic of our obviously failed attempt to be clear. Throughout the manuscript we will stick with stability and quality as the reviewer recommends.

Abstract...

L5: replace “rigorous” with something else, eg. “optimal”, or even “best”. Rigor is not being evaluated here. **L27:** replace “rigorous”. What is described in this paper is that the de novo methods produce OTU clusterings that coincide better with the pairwise distances between the sequencing reads. **L209:** Remove “rigorous”. You’ve used “accuracy” in this section (which appears identical to “quality” elsewhere, consider consolidating), so replace rigorous with accurate.

As suggested, we have replaced rigor/rigorous with optimal and accuracy with quality throughout the manuscript.

L7-L8: Recommend introducing “quality” here alongside its initial operational definition.

Thank you for this recommendation and it has been incorporated.

L16: replace “robust”. Robust is a particularly poor choice of word here, as it brings to mind stability, which is not what is being referred to. L245: Remove “robust”. The de novo methods produce clusterings that are more consistent with the pairwise separations of the individual sequencing reads. That does not correspond to any normal usage of the word robust. L362: replace “robust” L364: replace “robust”

As suggested, we have replaced robust throughout the manuscript

***L20-L21: USEARCH -> USEARCH and VSEARCH. According to your results, both methods produced OTU assignments that depended on the ordering of the reference database (~55-60% label consistency between reorderings of the database).**

We have corrected the text to indicate that both programs are deficient in this regard.

Introduction...

L46-47: Move the last sentence of this paragraph to the first sentence of the next paragraph.

We would prefer to leave this sentence where it is because it introduces the following three paragraphs.

L57-59: This sentence does not follow from the previous (don’t need “Therefore,”) and is not necessarily a shortcoming, but is rather an observation. All methods other than complete-linkage can join sequences together that are are less than 97% similar.

This has been edited as suggested.

L60: seuquence -> sequence. And drop “subtle”.

This has been edited as suggested.

***L64: The other major advantage of reference based methods is that the OTU assignments can be compared across experiments and even studies. This should be mentioned here.**

This has been edited as suggested.

L68: speed -> computational cost (or something similar)

This has been edited as suggested.

L76: drop “however”

This has been edited as suggested.

L80: Move last sentence ahead of previous sentence, as this follows from the reference independence property, not the ability to subsequently classify.

This has been edited as suggested.

L85: Clarify that this is a potential issue with “current” or “common” open-reference methods. It is not an inherent issue with any open-reference approach, as one can use coherent OTU assignment strategies for both parts (eg. join reference sequence or open-de-novo cluster if within 3%).

This has been edited as suggested.

***L95-105: Evaluation of accuracy on mock communities needs to be included in this discussion of previous methods to evaluate OTU methods.**

A sentence describing the use of mock communities has been added as suggested. Previously, these references were wrapped within the reference mapping approach.

L109: Those sequences that... -> Those pairs of sequence that... The unit of this analysis is pairs of sequences, not individual sequences.

This has been edited as suggested here and in the Methods section.

L117: attempted to characterize -> characterized

This has been edited as suggested.

L137-139: Either explain how the stability MCC is calculated, or reference the Methods at this point.

This has been edited by referring the reader to the Methods section.

L141: Multiple subsamplings was -> were

This has been edited as suggested.

L148-152: This is almost an extreme level of disagreement on this point between essentially identical AL analyses in the He study and this study. I find the He results rather unbelievable, but can the authors shed any further light on what might have happened? The He study doesn't provide a full workflow, but did provide the command they executed: `cluster(column=unique.dist,name=names,cutoff=0.05,precision=1000,method=average)`

My suspicion is that they did not compare apples-to-apples. I suspect they may have compared OTUs generated at one threshold for the subsampled data to OTUs generated at a different threshold for the full dataset. But it could really be anything. I have sought to clarify several questions with He and it has been a rather difficult process and decided to not bother with this point.

***L182-190: Comparing OTUs on the basis of their representative sequence is not “specious” and the He analysis was not “so poorly designed”. The most abundant sequence in an OTU is a biologically meaningful object, and we would like to think that OTUs are biologically meaningful and not just abstract objects. This section needs to be removed, or rewritten with less polemic language and with an explanation of how comparing representative sequences can break down (for example for very low abundance OTUs).**

We appreciate the concern regarding our polemical language and have edited the text to be less confrontational.

L243: the assignments are highly reproducible -> the overall quality of the assignments are consistent. The reproducibility of the assignments themselves was not evaluated here.

This has been edited as suggested.

L246: Evalution -> Evaluation

This has been edited as suggested.

L246-249: I don't really understand this. Swarm implements single-linkage clustering, in a way that is very clever computationally, but that is still single-linkage clustering.

We have clarified the description of the method to indicate that it is a SL method that then breaks up the long chains the method generally produces with a user-defined parameter.

L251: method -> methods

This has been edited as suggested.

L326-328: It would be useful to the reader to include the number of V4 covering reference sequences here. L323-328: Numbers of sequences and numbers of pairs of sequences are mixed together here in a confusing fashion. It would be better to do one or the other (eg. *7,785 pairs of sequences* that were more than 97% similar... -> *XXX sequences* within 97% of another reference sequence...). This allows the reader to easily evaluate the fraction of the reference database this corresponds to.

The first point has been edited as suggested and we believe that this helps to clarify the second point.

L341-345: Is this referring to sequences duplicated over V4? Or over the whole 16S gene?

This has been edited to indicate that these numbers were over the V4 region.

L354: "It is worth noting that the entire design of the He study was artificial." - This is unnecessarily polemic. There are ways in which the He study was not ideal, but essentially every OTU evaluation article uses an "artificial" design. The points being made here stand on their own, and are only obscured by this kind of language.

We thank the reviewer for encouraging us to take a less polemic tone. We have restructured this paragraph to make our point more clearly without being argumentative.

L390-395: It appears to me that the dependence on the order of the reference database has the potential to introduce *systematic* rather than just random errors, which is somewhat troubling.

We agree and have inserted a sentence here to underscore the point.

Reviewer 2 (Thomas Schmidt)

Basic reporting

In their manuscript, ‘De novo clustering methods out-perform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units’, Schloss & Westcott report a series of quality benchmarks on several established and recent 16S sequence clustering approaches and methods. The authors critically reproduced and extended a recent study by He et al. (Microbiome, 2015); they provide arguments and data that OTU “stability” is not necessarily indicative of OTU “quality”; they provide OTU quality benchmarks for very recent algorithms/software (SWARM and VSEARCH); and they report a novel perspective and tests on reference-based OTU demarcation. I believe that this work has merit, that the science is generally sound (but see comments below) and that the manuscript is timely and important for the growing community of microbial ecologists.

My main concern is that the study design is somewhat disparate, as the individual reported findings are not inherently inter-connected (see also comments on “Validity of the Findings”). Also, while the data and results provided generally support the authors’ conclusions, I am not convinced that benchmarks based on the Matthew’s Correlation Coefficient alone are the most informative approach for all the tests conducted (again, see comments below). Finally, there are several issues regarding the general reporting in the manuscript that could make it hard to follow for non-expert readers.

Following the journal's recommendation, I have tried to organize all comments into the appropriate sections ("Basic Reporting", "Experimental Design" and "Validity of the Findings"). Within these sections, I tried to organize all major comments in decreasing order of importance and minor comments chronologically, following the manuscript. However, several points touch more than one of the specified comment sections, and I have tried to disentangle these as much as possible in order to achieve a readable review.

Comments on "Basic Reporting".

- The manuscript is overall very readable, clearly written, the global structure is clear and all individual sections are well structured. That being said, the structure of the study is also somewhat disparate: the individual parts stand somewhat independently from each other and do not form a fully coherent body of research. In the current form, the authors do not make it sufficiently clear how the first part of the manuscript (a critical reproduction of the study by He et al) relates to the second (quality benchmarks of the very recent methods SWARM and VSEARCH) and, in turn, to the third (general problems with reference-based OTU demarcation). While all of these findings are valid and interesting in themselves, the manuscript seems to be telling several unrelated stories at once. One general topic is a comparison of de novo vs reference-based OTUs, another is the question of OTU "stability" vs "accuracy" and a third thread refers to the clustering quality of novel methods, in particular a comparison of USEARCH to its open-source clone VSEARCH. As I said, the manuscript is very readable as is, but these findings are not sufficiently synthesized in the Conclusions, in my opinion.

The reviewer raises a great point. We have added a paragraph at the end of the section where we describe our reproduction of the He study. The paragraph lays out five additional questions that we raised because of this reproduction analysis. These questions now serve as the sub-section headings for the next five sub-sections of the Results & Discussion section. We are optimistic that this paragraph helps to tie together the various parts of the analysis.

- All Figures, in their current form, are really non-obvious to interpret. Frankly speaking, when I first read through the manuscript, I understood all results from the text but really struggled at interpreting the figures. I believe they would profit from a careful workover.

We appreciate the reviewer's feedback and have altered the presentation of the figures and their captions. Hopefully, their presentation is more clear in the current draft.

=> Figures 1&3 lack color legends (it took me some time to realize that colors in the left panel corresponded to the categorical axis in right panel). Also, being partially color-blind, I had a hard time distinguishing the thick lines in Fig 1&3, left panels. Ordinate axis labeling in Fig 1&3 was not intuitive on a first read-through; the meaning of "MCC relative to full dataset" and "to distances" was not clear, and only a close look at the corresponding Methods section (but not the Figure caption) helped resolve this.

We have added a plotting symbol to the lines in panels A and C. These plotting symbols have been used in panels B and D. Because the lines in A and C overlap, there is a risk that including plotting symbols for all of the fractions would be impossible to read. To make lines easier to differentiate in A & C we have made them thinner. In addition, we have re-labeled the y-axes as Stability and Quality and then defined those terms in the figure caption.

=> In my opinion, Fig 2 presents the data in a non-intuitive way. I am not sure which point the authors are trying to emphasize here: the illustration draws the focus to the generally varying number in absolute OTU counts, but from the arguments in the text I believe that relative differences in counts between re-clustered sub-setted datasets and rarefied full datasets are more important. The latter, however, are not clear to interpret from the figure as is. Also, it is not clear to me why clustering methods are the categorical axis while down-sampling steps are scattered vertically. Moreover, a comparison of SWARM is missing from this figure for some reason (?). I would suggest to split this figure into two parts: part A providing more classical rarefaction curves per method (with sub-setted clustering points as overlay scatter) and part B providing the (log) relative OTU count between rarefaction and sub-setting across down-sampling steps.

We have added the Swarm data to this plot. Although we see the reviewers point of presenting the absolute difference between the sub-sampled and rarefied datasets, we also wanted to present the difference in the inferred richness using the different methods. As the reviewer indicated, we could present traditional rarefaction plots, but this would result in a figure with 8 panels. We feel that this presentation does the best job of showing that there really is little difference between the subsampled and rarefied data for most methods and that there are large differences in the number of OTUs generated by the various methods.

=> Figure 4 lacks a caption that is explanatory beyond the figure title.

Similar to the previous comment, we have changed the Relative to full dataset / Relative to distances descriptions to Stability and Quality and defined these terms in the figure caption. We also expended the description of the plot.

- The Introduction is generally very clear, concise and readable. However, there is a large body of previous work on testing methods for OTU clustering, and the authors focus on only a very limited subset of studies. While I think that a comprehensive review of all studies on OTU benchmarking ever published is certainly outside the scope of the present work, I believe that the manuscript would much profit from a more in-depth discussion of at least some. Other works possibly worth mentioning include, but are not limited to: => Huse et al., Env Microbiol, 2010, DOI: 10.1111/j.1462-2920.2010.02193.x => White et al., BMC Bioinformatics, 2010, [doi:10.1186/1471-2105-11-152](https://doi.org/10.1186/1471-2105-11-152) => Barriuso et al., BMC Bioinformatics, 2011, [doi:10.1186/1471-2105-12-473](https://doi.org/10.1186/1471-2105-12-473), who indeed provided an early investigation into “output variability” of OTU clustering (Table 7) => Bonder et al., Bioinformatics, 2012, doi: 10.1093/bioinformatics/bts552 => Koeppel & Wu, NAR, 2013, doi: 10.1093/nar/gkt241 => Schmidt et al., PLOS Comp Biol, 2014, DOI: 10.1371/journal.pcbi.1003594 => May et al., Bioinformatics, 2014, doi: 10.1093/bioinformatics/btu085 However, these are merely suggestions and the authors should not feel obliged to include any or all of these as new references in the Introduction – in particular, since I also pointed out one work of which I am co-author. Nevertheless, I do believe that the manuscript would generally profit from a discussion of (some) further previous work.

- The Introduction provides a detailed, accessible and balanced explanation of the three general approaches to OTU demarcation discussed in the paper: *de novo*, closed reference and open reference. Although the authors point to two comprehensive references which give further information on these points (Schloss & Westcott, 2011; Navas-Molina et al., 2013), I would suggest that the authors provide at least some visual/structural guidance to the reader (an overview figure or table). However, the manuscript is certainly understandable as is, so please consider this comment as a mere suggestion.

We appreciate the reviewer's suggestions for additional papers to cite in our Introduction. Many of these have been added to the text as appropriate. As the reviewer can appreciate there have been many papers published in this area of varying quality. We did consider developing an overview figure or table to describe the various methods, but realized that the text was quickly becoming a review article. In addition, if this paper is read in conjunction with the original He study, much of the material is already covered in their study and we did not feel it was necessary to recover the same material.

- Related to this, the terms “abundance-based” and “distance-based greedy clustering” (AGC and DGC) are not properly introduced. Although the authors point out that they stick with the terminology of the He et al paper, these terms need to be explained somewhere in the manuscript, preferably in the Introduction.

We have added text in the paragraph describing *de novo* approaches defining AGC and DGC.

- Similarly, the last part in the Results and Discussion, “Problems with reference-based clustering [...]”, could profit from an illustration. This part is very clearly written, and the presented results are striking (see also comments under “Validity of the Findings”). Nevertheless, a visual presentation of the data could additionally help to guide the reader through the conducted tests and illustrate just how striking the reported results are. Even a simple table summarizing the results would be helpful.

We thank the reviewer for this recommendation. We have added a new figure showing the distribution of OTU numbers when the databases are randomized for both USEARCH and

VSEARCH. We also show the expected number of OTUs based on calculating the actual distances between the murine dataset sequences and the references.

- The authors very clearly point out weaknesses in the criticized study by He et al; they support all their arguments with data and/or sound reasoning and do not, in my opinion, state any unfair or unsupported claims against the criticized work. Nevertheless, I personally felt that the wording is sometimes a bit harsh: e.g., p.9, l.190 (“this analysis was so poorly designed”); p.15, l.354 (“the entire design of the He study was artificial”).

Thank you for this comment, we have edited these sections to be less confrontational.

Minor comments:

- Please re-check for typos and grammar across the text (e.g., p.5, l.60 “seuquence”; p.5, l.69 “scale[s]”; p.6, l.88 “assigned”; p.7, l.137 “assess the calculated the MCC”; etc.)

Thank you for pointing out these errors. We have corrected these and have gone back through the text to identify any additional problems.

- Several examples given throughout the text appear rather arbitrary. For example, p.5, l.59 (“may only be 94% similar”, same in p.17, ll.402-3) or p.6, l.89 (“all sequences assigned to the Porphyromonadaceae would then be assigned to OTUs [...]”) are out of context, the sudden mention of a concrete clustering threshold or an arbitrary bacterial family could confuse the reader more than illustrate the point being made.

On the first point, the 94% similar follows from the previous sentence indicating that two sequences could be 97% similar to a reference. The 94% would be a worst case scenario where those two sequences are similar to the reference, but only 94% similar to each other (i.e. $100 - 3 - 3 = 94$). In the second case, this is described as an example of a hybrid approach. We have edited the text around these comments to hopefully make these points more clear.

- p.4, l.56. I believe that the provided reference for the statement that “the commonly used variable regions [...] do not evolve at the same rate” is not fully appropriate. Citing Schloss, Plos Comp Biol, 2010 here (as is done elsewhere in the text) seems more

meaningful, as this study is arguably the most comprehensive to date regarding this point. Moreover, it is probably also worth mentioning the related contribution by Kim et al. (Journal Microbiol Methods, 2011, <http://www.ncbi.nlm.nih.gov/pubmed/21047533>).

This has been edited as suggested.

- When enumerating the pros and cons of closed ref clustering (p.5, ll. 48f), several points in favor of this approach could be added: it is trivially parallelizable, has a low memory footprint and arguably gets better as reference databases improve (which is the case as more and more sequence data accumulates).

This has been edited as suggested.

- p.6, l.93-4. This statement is phrased too strongly. The approach advocated by the authors (taxonomic classification, followed by de novo clustering within taxonomic bins) has been shown to be very useful in practice, but presenting it as an ideal compromise between closed reference and de novo approaches is an overstatement and potentially misleading. The authors, themselves, point out just a few lines later that taxonomic classification is often far from perfect (it could be added that it depends strongly on reference database quality and classifier performance); however, if the classification step is not perfect, the entire clustering outcome will be (strongly) affected. Others have suggested database-agnostic alternatives in a similar spirit, such as SL pre-clustering (Huse et al., Env Microbiol, 2010) or the kmer-pre-clustering implemented in ESPRIT-Tree (Cai & Sun, NAR, 2011) which would be worth mentioning here.

We have added a clause to the end of this paragraph indicating that our previously proposed hybrid approach still suffers from database quality and classification errors.

- p.6, ll.109ff. This paragraph explaining the MCC (in detail) feels a little out of place here; I suggest to relocate it to the (early) Results section.

Thank you for the suggestion. Because we needed to reorganize the text, the details have been removed from the Introduction and are now in the Methods, which precedes the Results & Discussion.

Experimental design

- Methods are reported clearly and concisely, links to available data are provided, all tested tools are specified down to the version number. More Methods sections should look like this! In particular, I would like to commend the authors for uploading all code as reproducible workflow to Github.

Thanks! We try to lead by example :).

- Matthew's Correlation Coefficient (MCC) is used throughout the study as the sole indicator of OTU "quality". The MCC is an objective summary statistic for classification problems and has been successfully used for OTU benchmarking before (originally introduced by the authors in their 2011 paper and since then picked up by several other teams). Nevertheless, I would like to point out three general objections to using the MCC as an OTU quality criterion and then argue that the MCC, when used as a sole index, is not the most informative choice for (all) the questions that the authors pursue in this study.

=> In general, the MCC is a summary statistic for binary classification problems. However, (OTU) clustering is arguably not a problem that boils down to binary true/false assignments only. Of course, violations of a "hard" sequence similarity clustering threshold provide an objective criterion, but different algorithms implement inherently different concepts of a "threshold", and are consequentially prone to different types of errors. For example, by definition, complete linkage or USEARCH would never show "false positive" clustering (but many false negatives can be expected), while single linkage would never make "false negative" mistakes (while a wealth of false positives is expected). In other words, the MCC was not designed for this kind of application and OTU "quality" is not (only) a binary classification problem; nevertheless, MCC values are arguably informative, if interpreted carefully. => My second "general" issue with the MCC concerns its formulation as a

summary statistic: frankly speaking, I find it rather non-intuitive to interpret. For example, in the present study, it would be very informative to see what actually goes “right” or “wrong” for the different methods, driving MCC values (e.g., by showing distributions of TPR, FPR, etc. per clustering method). => Finally, I suspect that the MCC is sensitive to cluster count and size distribution, although I am not aware that this has ever been tested. In general, more “errors” can be expected the further a clustering algorithm proceeds: sets with fewer and larger OTUs would be more prone to erroneous assignments than sets with many small OTUs. This is problematic, because the “hard” clustering threshold is implemented very differently by different algorithms, and it is well established that at a given threshold of, say, 97% the different methods provide very different cluster count and size distributions. In the present study, such effects would be especially striking for the SWARM algorithm.

In spite of these comments, the use of the MCC in the second use case of the present study (“MCC value relative to distances”) is arguably established and meaningful, if interpreted cautiously. However, in my opinion, the MCC is an insufficient metric in the first use case (“MCC value relative to full dataset”). The authors generated “reference” OTU sets based on the full datasets and subsequently compared OTU sets based on randomly down-sampled datasets to these using the MCC. In this setup, TPs are sequence pairs which end up in the same OTU under full and down-sampled clustering, etc. Arguably, this corresponds even less to the original use case of the MCC, and putative differential cluster size and count effects would be even more striking (based on the authors’ findings in Figure 2). What appears particularly confusing is that the interpretation of the MCC changes drastically between both use cases: the “MCC on distances” is interpretable in line with previous work, but the “MCC relative to full dataset clustering” means something completely different.

Several metrics have been developed which appear more appropriate for the latter use case. The down-sampling experiment, in my opinion, would be more straightforward to interpret if clustering comparison metrics, e.g. based on mutual information were to be used. I freely admit to a “conflict of interest” here: in a previous work I was involved in (DOI:

10.1111/1462-2920.12610), we used Adjusted Mutual Information (Vinh et al., 2010, <http://www.jmlr.org/papers/v11/vinh10a.html>), Normalized Mutual Information (Fred 7 Jain, 2003, <http://ieeexplore.ieee.org/stamp/stamp.jsp?arnumber=1211462>) and the Adjusted Rand Index (Hubert & Arabie, 1985, <http://link.springer.com/article/10.1007%2FBF01908075>) in a very similar setup (testing clustering robustness to selective down-sampling). While I generally consider it very impolite to push one's own work as a reviewer, I do believe that in this case, there is a clear scientific motivation for doing so. For the tests conducted in Fig 1AB & 3AB, at least, I believe that the above measures would provide a highly informative complement, if not replacement, for the MCC. They are straightforward indices of OTU “stability”, in the sense defined in the present manuscript. Indeed, based on our previous observations (Fig 5 in Schmidt et al., Environ Microbiol, 2015), I would expect that the trends observed in the present study would be even more pronounced. Taken together, MCC and AMI/NMI/ARI could then indeed be interpreted as OTU “accuracy” and “stability”. Likewise, the comparison between USEARCH and VSEARCH is a model use case for which the above-mentioned methods were originally designed.

I appreciate the reviewer's comments and can judge from their comments elsewhere in this review that they were a bit on the fence about whether to recommend we use the ARI/NMI/AMI in place of the MCC for the measurement of stability in the first part of the manuscript where we try to replicate the He paper's work. Our initial inclination is to stick with the MCC approach, which was the method used by the original He study. If we were to do one of the other methods instead, then I feel like that would confuse the issue and would be a different type of replication. I feel that indicating that they perhaps picked the wrong metric would add unnecessarily to the figures and text when the point of the first section of the Results section is that their idea of “stability” is subservient to the quality of OTU assignments. Compounding this complexity would be the decision to pick one or all of the three choices to replace or supplement MCC for measuring stability. Furthermore, we feel that the consistency of that quality over multiple random seeds is much a better metric of “stability” than to compare the subsampled OTU assignments to the full dataset assignments. That being said, I feel like we've done a good job of addressing the quality/stability issue and the reviewers both seemed to agree. If the reviewer and editor feel that this would be an important issue to incorporate

into the manuscript, then we'll do it. At this point, it just seems like piling on when we've already made the point that their concept of "stability" is problematic. Finally, we have added a sentence to the section of the Results section where we attempt to replicate the He study indicating that information-based methods have previously been used and are likely better, but that we chose to stick with the original approach.

To the point of interpreting the MCC value, we have added a sentence to the end of the penultimate paragraph in the Introduction that provides an interpretation.

Validity of the findings

- As detailed above, I believe that all conclusions are generally justified by the analyses conducted, albeit reservations regarding the use of the MCC as sole index in the stated cases. However, as likewise detailed above, I also believe that several findings stand independently of each other, and their connections are not immediately obvious.

We hope that the inclusion of the paragraph to the Results & Discussion section outlining the questions that were raised by our replication of the He study provides a sufficient roadmap to help tie the paper together better.

- One main rationale of the present study is that OTU assignment "accuracy" trumps "stability". The authors provide clear reasoning to support this argument, and their data is generally in line with this notion. Nevertheless, one could argue that indeed, both "stability" and "accuracy" are important features of OTUs, and that their relative significance depends on the application. It is neither desirable to work with "stable but wrong" nor "accurate but noisy" OTUs. I agree with the authors that accuracy is the more desirable property in general, but putting their study in context with other recent findings could suggest that in fact, accuracy and stability tend to be correlated, which would lend even more gravity to their argument.

We agree that some metric of stability is important. We feel that by running the methods 30 times on the same dataset and essentially getting the same quality value (i.e. MCC) relative to the

distances between sequences, is a superior metric of stability than comparing the subsampled OTU assignments to those from the full dataset.

- The points raised in critical response to the He et al. study are all well illustrated, argued and supported by data. Moreover, by repeating several of the tests on a more recent (and more reasonably chosen), more deeply sequenced dataset, the authors provide a clear added value.

- The inclusion of tests on the very recent SWARM and VSEARCH algorithms makes this manuscript very timely and useful for researchers in the field. To my knowledge, this is the first available independent test on VSEARCH performance.

- The findings on “problems with reference-based clustering in general and as implemented in QIIME” are striking and, indeed, slightly disturbing. To be honest, I would not have expected such strong effects of sequence order for mappings against the QIIME reference database. I believe that these results will contribute to more debates in the field, and ultimately lead to further long-needed benchmarks on standard operating procedures for widely used software.

Thank you for highlighting these three points.

- p.12, ll.260-1. This statement is too harsh based on the given data. The SWARM algorithm is designed to not work with “hard” thresholds in the classical sense, so it is not surprising that in the given testing framework, different “thresholds” would appear optimal for differentially deeply sequenced datasets.

We attempted to soften this language. Regardless, in our opinion, the inability to define a “hard” threshold makes it difficult to compare results across studies.

- p.15, ll.368-9. This solution appears slightly impractical. While it would clearly be desirable if researchers provided clustering quality metrics along with their results (and ideally, for more than one clustering method), calculating the MCC would always require to calculate and store full sequence distance matrices. Also, it is unclear what an

MCC comparison across different studies would actually mean (considering additional confounding factors such as sequencing depth, clustering threshold, alignment, etc.). Nevertheless, I fully agree with the authors that researchers should ideally perform (and report) different clusterings on their data.

We attempted to soften this language.

Comments for the author

I would like to again commend the authors on their manuscript and again apologize for pushing a study their way in which I was involved. I was very hesitant to include the points on the use of the MCC vs AMI/NMI/ARI in this review, but I honestly believe that they make sense scientifically.

Thank you for your openness and thoroughness. Both reviews have gone a long way to improving the quality of the manuscript.