Reintroducing mothur: 10 years later

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Mini-review

Abstract

250 words

Importance

150 words

Few scientists set out on a nearly two decade-long journey with a specific goal in mind. Often we fail to start a scientific journey because it looks too hard. Perhaps we get discouraged by all of the things that could go wrong. Maybe we stray from the path because we find something that is more interesting. Every scientist picks their own path and takes their own forks in the road. From the outside, it may appear to be a random walk. Nevertheless, these meanderings are common in science.

Looking back on scientific journeys can be instructive to others who are overwhelmed at the prospect of looking forward at their careers (1–5). By no means is my personal journey over, but since 2002 I have been on a journey that I did not realize I was on. Now that the paper introducing the mothur software package is ten years old and has become the most cited paper published by *Applied and Environmental Microbiology* (7), it is worth stepping back and using the continued development of mothur as a story that has parallels to many other research stories.

I fondly recall preparing a poster for the 2002 meeting of research groups supported by the NSF-supported Microbial Observatories Program. I wanted to triumphantly show that I had sequenced more than 600 16S rRNA gene sequences from a single 0.5-g sample of Alaskan soil. This was greater sequencing depth than anyone else had achieved for a single sample. As I was preparing the poster, I walked into the office of Jo Handelsman, my postdoctoral research advisor, and laid out the outline for the poster. She asked if I could add one of those “curvy things”, a rarefaction curve, to show where I was in sampling the community. Rarefaction curves and attempts to estimate the taxonomic richness of soil had become popular because of the impactful review by Jennifer Hughes and her colleagues (8). Their seminal paper introduced the field to operational taxonomic units (OTUs), rarefaction curves, and richness estimates. I do not recall whether my poster had a rarefaction curve on it, but Jo’s question primed my career.

Introducing DOTUR and friends. When Jo asked me to generate a rarefaction curve for the poster, the request was not trivial. How would I bin the sequences into OTUs? Hughes and her colleagues did it manually and with fewer than 300 sequences. Although I could possibly do that for my 600 sequences, my goal was to generate 1,000 sequences from the sample and to repeat that sampling effort for other samples. I needed something that could be automated. Furthermore,

the software that Hughes used, EstimateS (4), required a series of tedious data formatting steps to perform the analyses we were interested in performing. I had found my first problem. How would I assign sequences to OTUs and use that data to estimate the richness and diversity of a sample? The second problem would involve comparing the abundance of OTUs found in one sample to another sample. The solution to the first problem, DOTUR (Distance-based OTUs and Richness), took us two years to develop (9). DOTUR did two things: given a matrix quantifying the genetic distance between pairs of sequences, it would cluster those sequences into OTUs for any distance threshold to define the OTUs and then it would use the frequency of each OTU to calculate a variety of alpha diversity metrics. The solutions to the second problem would come from our work to develop software including $\int$-LIBSHUFF (10), SONS (Shared OTUs and Similarity) (11), and TreeClimber (12). Around the same time, Catherine Lozupone and Rob Knight were developing their UniFrac tools to compare communities with a phylogenetic rather than OTU-based approach (13, 14). With these tools, the field of microbial ecology had a quantitative toolbox for describing and comparing microbial communities. Along the way Jo and I would demonstrate the utility of such tools to answer questions like how many OTUs were there in that sample of Alaskan soil and how many sequences were needed to sample each of those OTUs (15)? Where were we in the global bacterial census (16)? How does the word usage of *Goodnight, Moon* compare to that of *Portrait of a Lady* and more importantly how is this relevant to microbial ecology (17)? Most edifying were the more than 2,400 papers that used DOTUR, SONS, TreeClimber, or $\int$-LIBSHUFF to facilitate their own research questions (Web of Science, 10/1/2019). Had we waited to solve all of the problems that plagued 16S rRNA gene sequencing, we would still be waiting.

It is important to remember that we knew there were many problems with 16S rRNA gene sequencing. We knew there were biases from extractions and amplification (18–23). We knew there were chimeras (24–27). We knew that bacteria varied in their *rrn* copy number. Generating a distance matrix was a prerequisite to using my tools. This wasn't trivial, but by cobbling together other tools it was possible. We would assemble, trim and and correct Sanger sequence reads using Chromas or STADEN (28), align the sequences using ClustalW (29) or ARB (30), check for chimeras using partial treeing or Bellerephon (27), and calculate a pairwise distance matrix using DNADIST from the PHYLIP package (31). At the time, we knew that we only had a loose

concept of a species based on these distances (32). We hoped that an OTU defined as a group of sequences more than 97% similar to each other would be a biologically meaningful unit regardless of whether it fit our notion of a bacterial species. At the time, I felt that the biggest problems that I could solve were how to cluster the sequences into OTUs and how to use those clusterings to test our hypotheses. The only tool available at the time that automated the clustering step was FastGroup, which implemented a greedy version of the single linkage algorithm (33). The high cost of sequencing was also an impediment to experimentation and analysis in microbial ecology. It was rare for a study design to have experimental replicates so that one could perform a statistical test to compare treatment groups. For example, in our testing we frequently used a dataset comparing Scottish soils from from Alison McCaig and colleagues (34). This dataset consisted of two experimental groups, each replicated three times with 45 sequences per replicate. Although great focus has been placed on the depth of sampling afforded by 454 and Illumina sequencing, the true benefit of the modern sequencing platforms is the ability to affordably sequence a large number of technical and biological replicates. In spite of the many technical challenges, we had excuses and heuristics to solve problems that served our needs. It is telling that a recent review of "best practices" in generating and analyzing 16S rRNA gene sequences shows that we still have not solved many of these issues and that we have even identified additional problems (35).

As we developed these tools, I found a unique niche in microbiology. My undergraduate and graduate training as a biological engineer prepared me to think about research questions from a systems perspective, to think quantitatively, and to understand the value of using computer programs to help solve problems. As an undergraduate student, I learned the Pascal programming language and promptly forgot much of it as a graduate student and learned MatLab in its place. As a postdoc, I learned the Perl programming language to better understand how LIBSHUFF, a tool for comparing the structure of two communities, worked since it was written in Perl (36). After writing my own version of LIBSHUFF, $\int$-LIBSHUFF and seeing the speed of the version written in C++ by my collaborator, Bret Larget, I converted my Perl version of DOTUR into C++. At the time, the conversion from Perl to C++ seemed like an academic exercise to learn a new language. My Perl version only took a minute or so to process the final collection of 1,000 sequences and the

C++ version took seconds. Was that really such a big difference? In hindsight, as we now process datasets with millions of sequences, the decision to learn to C++ was critical. The ability to pick up computer languages to solve problems was enabled by my prior training. It was also a skill that was virtually unheard of in microbiology. Today, researchers without the ability to program are at a significant disadvantage (37).

Introducing mothur. Shortly after DOTUR was published, I received an email from Mitch Sogin, a scientist at the Marine Biology Laboratory (Woods Hole, MA), who asked whether DOTUR could handle more than a million sequences. Without answering his question, I asked where he found a million sequences. Little did I know that his email would represent another pivot in the development of these tools. His group would be the first to use 454 sequencing technology to generate 16S rRNA gene sequences (38). Although DOTUR could assign those sequences to OTUs, at the scale of millions of sequences, it was slow and required a significant amount of RAM. As I left my postdoc to start my independent career across the state from Sogin's lab at the University of Massachusetts in Amherst, my plan was to rewrite DOTUR, SONS, $\int$-LIBSHUFF, and TreeClimber for the new world of massively parallelized sequencing. The new tool would become mothur.

Milling about at a poster session at an ASM General Meeting in New Orleans, I ran into Mitch who asked what my plans were for my new lab. I told him that I wanted to make a tool like ARB, a powerful database tool and phylogenetics package (30), but for microbial ecology analysis. His retort was, "You and what army?" Up to that point, I had written every line of code and been answering many emails from people asking for help. He was right, I would need an army. It would be difficult, but I needed to learn to let go and share the development process with someone else. My "army" ended up being Sarah Westcott who has worked on the mothur project from its inception. Today, mothur is over 200,000 lines of code and Sarah has touched or written nearly every line of it. Beyond writing and testing mothur's code base, she has become a conduit for many who are trying to learn the tools of microbial ecology. She patiently answers questions via email and on the package's discussion forum (https://forum.mothur.org). The community and I are lucky that Sarah has stayed with the project for more than a decade. To be honest, such dependency on a single person makes the project brittle. In hindsight, it would have been better to have developed mothur with more of an "army" or team so that there is overlap in people's understanding of how

mothur works. Although a distributed team approach might work in a software engineering firm, it is not practical in most academic environments where there is limited funding. There are certainly projects that make this work, but they are rare.

Competition has been good and healthy. mothur has not been developed in a vacuum and it does not have a monopoly within the field. As indicated above, each of our decisions were made in the historical context of the field and with constant pressure from others developing their own tools for analyzing 16S rRNA gene sequence data. Competition has been good for mothur and for the field.

From the beginning there have been online tools available at the Ribosomal Database Project (RDP) (39), greengenes (40), and SILVA (41). These allowed users a straightforward method of comparing their data to those collected in a database. There are two primary downsides to these tools. First, researchers running the online tool must pay the computational expenses leading to slow process times when hardware becomes outdated and when numerous users simultaneously attempt their analysis. Eventually this limitation would result in the termination of the greengenes website. Second, these platforms provide a one-size-fits-all analysis. These tools only allow a user to analyze 16S and in some cases 18S rRNA gene sequences. If a user sequences a different gene, then the tool will not serve them. These observations resulted in two design goals we have had with mothur: bringing the analysis to a user's computer and separating a tool from a specific database. For example, we commonly use a sequence alignment method that was originally developed for greengenes (42), but use a SILVA-based reference alignment because its superior quality (43, 44). In addition, we offer the naïve Bayesian classifier developed by the RDP (45) and allow users to train it to any database they want, including customized databases. In both examples, users can align or classify non-rRNA gene sequence data. As the bioinformatics tools have matured, both the RDP and SILVA offer integrated pipelines for analyzing large datasets, albeit in one-size-fits-all black box implementations.

With the growth in popularity of 16S rRNA gene sequencing there has naturally been an expansion in the number of people developing tools to analyze these data. Months after the paper describing mothur was published, the paper describing QIIME was published (46). Over the past 10 years,

many have attempted to create analogies comparing the two programs: Pepsi vs Coke, Apple vs Windows, etc. It is never clear which software is which brand and whether the comparisons are meant as a complement or an insult. Regardless, both programs are very popular. From my perspective, most of the differences are cosmetic. QIIME is effectively a bundle of scripts to run other developers' software. For example, with QIIME (through version 1.9.1), it was even possible to run mothur through QIIME. One can also run the naïve Bayesian classifier through QIIME using the original code developed by the RDP. This caused great frustration for many users because there were numerous software dependencies that had to be installed. Although the QIIME developers would go on to create virtual machines and use packaging tools to simplify installation, these fixes required sophistication by users who we knew struggled with the basics of navigating a command line. In contrast, when a user runs mothur, they are running mothur. The naïve Bayesian classifier code that is in mothur is a rewritten version of the original code. When we rewrite someone's software we do it with an eye to improving performance, access, and utility for non-16S rRNA gene sequence data. For example, while 454 data was popular, PyroNoise was an effective tool for denoising flowgram data (47). Running the original code required a large Linux computer cluster and knowledge of bash and Perl scripting. When we rewrote the code for mothur, we made it accessible to people using any operating system with a simple command interface (i.e. trim.flows and shhh.flows). Our approach requires significant developer effort, but saves considerable user effort. As this benefit is multiplied across thousands of projects, the savings to users has been considerable.

Beyond the large packages like mothur and QIIME, there has been significant growth in stand alone software tools for sequence curation (e.g. PyroNoise (47), PANDAseq (48), DADA2 (49)), chimera checking (e.g. UCHIME (50), ChimeraSlayer (51), Perseus (52)), and clustering (e.g. USEARCH (53), VSEARCH (54), Swarm (55)). Where possible and when warrented, we have implemented many of these algorithms directly into mothur. We have also used this diversity of methods to perform head-to-head comparisons. Most notable is the area of clustering algorithms where there have been a large number of algorithms developed without an obvious method to objectively compare them (56–59). We applied an objective metric, the Matthew's Correlation Coefficient (MCC), to evaluate numerous algorithms for clustering sequences into

OTUs. By performing this type of analysis, we were able to objectively compare the algorithms, make recommendations to the field, and develop new algorithms that outperformed the existing algorithms. Beyond evaluating clustering algorithms, we have also evaluated methods of denoising sequence data (60–62), assessed reference alignments (43, 44), considered the importance of incorporating secondary structure information in alignments (63), quantified the variation along the 16S rRNA gene (44), and compared the statistical hypotheses tested by commonly used tools (64). We have embraced the competition and diversity of all methods being used to analyze amplicon data as an opportunity to identify the strengths and weaknesses of the methods in an attempt to make recommendations to other researchers.

mothur's core principles. As mothur has evolved with the needs of the community, several core principles have emerged that direct its development. First, mothur is a free, open source software package. This has been critical in shaping the direction of mothur. We were content for mothur to be an improved combination of DOTUR and SONS and leverage existing tools for other steps. Yet, when we learned that the code for NAST, the algorithm behind the greengenes's aligner (42), was not open source or publicly available. Similarly, although the SINA aligner was available through ARB and the SILVA website performed well it was closed source. Because the ARB implementation did not scale to large datasets, researchers were left to pay a processing fee to the SILVA website to align sequences. Thus, we realized that such an important tool needed to be opened to the community (43). More recently, the rejection of closed source, commercial tools such as USEARCH can be seen by the broader adoption of its open source, free competitor, VSEARCH, within the microbial ecology community (53, 54). Related to insuring that mothur's code is open source, our second core principle is that we maintain transparency to our users. Perhaps a user does not need to interrogate every line of code, but they need to understand what is happening. Many programs including online workflows encapsulate large elements of a pipeline in a single command. In contrast, mothur forces the user to specify each step of the pipeline. Although the former approach makes an analysis easier for a beginner, it potentially stifles users that need greater control or understanding of the assumptions at each step. This control over the pipeline has made it easier for researchers to customize databases or adapt the pipeline to analyze non-16S rRNA gene sequence data. Third, as I mentioned above, there has been a plethora of

methods proposed for generating amplicon sequence data, and curating, aligning, checking for chimeras, classifying, and clustering the data. I am proud of the data-driven approach we have taken to comparing these methods. A description of a new method is of limited value if it is not benchmarked against other methods or control datasets. Through this core principle and mothur's large reach into the community, we have helped to develop standards in the analysis of 16S rRNA gene sequence data. Fourth, a focus on enabling reproducibility has always been central to the functionality of mothur. From the beginning, mothur's logfiles have represented a transcript of the user's command and outputs. When researchers were reluctant to submit sequence data to the Sequence Read Archive (SRA), we worked with the SRA developers to create a mothur command (make.sra) to create the package to submit sequence data through a special mothur portal. A more ambitious project had its seed on April 1, 2013 when we announced a new "function" in mothur: write.paper. The new command required that the user provide a 454 sff file and a journal title or impact factor. With this information, mothur would generate a manuscript. This April Fools' Day joke was poking fun at software that provided an analysis black box but also at many users' sentiments that data analysis should be so cut and dry. A few years later, we revisited this concept in the scope of reproducibility. Why not explicitly script an analysis from downloading data from the SRA through the rendering of a manuscript ready for submission? This idea gave rise to the development of the Riffomonas reproducible research tutorial series that enables researchers to write their own version of write.paper (65). Perhaps the most important core principle is that my research group uses mothur to analyze the data we generate. We "eat our own dogfood". This has proven critical as it again represents transparency and hopefully provides confidence to mothur's users that we are not making recommendations that we do not follow ourselves.

Challenges of making open source count. Anyone can post code to GitHub with a permissive license and claim to be an open source software developer. Far more challenging is engaging the target community to make contributions to that code. Frankly, we have struggled to expand the number of people that make contributions to the mothur code base. One challenge we face is that if we looked to third parties to contribute code to mothur, they would need to know C++. Given the paucity of microbiologists with skills programing in a compiled language like C++, expecting that community to provide contributors that can write code in a syntax that prizes execution efficiency

over developer efficiency was not likely. In contrast, the QIIME development team could be more distributed because their code base was primarily written in Python, which prizes developer efficiency over execution efficiency. QIIME is a series of wrappers that allow users to execute other developers' code making the use of a scripting language like Python attractive. Their choices resulted in many tradeoffs that have impacted ease of installation, usability, execution speed, and flexibility. If we were offered funding to rewrite mothur, we would likely rewrite it as an R package that leaned heavily on the R language's C++ interface packages. Of course, such choices are always best in hindsight. Yet, when we started developing mothur, the ability to interface between scripting languages like R and Python and C++ code was not as well developed as it is today. For example, the modern version of the Rcpp package was first released in 2009 and its popularity was not immediate (66). Again, the development of mothur has been a product of the environment that it was created in. Although these decisions have largely had positive outcomes, there have been tradeoffs that caused us to sacrifice other goals.

Beyond contributing to the mothur code base, we sought out other ways to include the community as developers. The paper describing mothur included 15 co-authors, all but three (Schloss, Westcott, and Ryabin) responded to a call to provide a wiki page that described how they used an early version of mothur to analyze a data set. Our vision was that authors might use the mothur wiki to document reproducible workflows for papers using mothur but to also provide instructional materials for other seeking to adapt mothur for their uses (https://www.mothur.org/wiki). Unfortunately, once the incentive of co-authorship was removed, researchers stopped contributing their workflows to the wiki. Again, this vision and the lack of the community's adoption of wikis as a mechanism for reporting workflows was a product of the environment. Although wikis were popular in the late 2000's, they lacked the ability to directly execute the commands that researchers reported. Such technology would not be possible until the creation of IPython notebooks (2011) and R markdown (2012). Another problem with the wiki approach was that potential contributors did not see the wiki as a community resource. I frequently received emails from scientists telling me that there was a typo on a specific page when the intention was that they could correct the typos without my input. We have been more successful in soliciting input and contributions from the user community through the mothur discussion forum and GitHub-based

issue tracker. As mothur has matured, we have been dependent on the user community to use these resources to tell us what features they would like to see included in mothur and where the documentation is confusing or incomplete (https://forum.mothur.org). Often we can count on people not directly affiliated with mothur to provide instruction and their own experience to other users on the forum. We are constantly trying to recruit our "army" and are happy to take any contributions we can. Whether the contributions are to the code base, discussion forum, or suggestions for new tools, these contributions have been invaluable to the growth and popularity of mothur.

Failed experiments. If we never failed, we would not be trying hard enough. Over the past decade we have tried a number of experiments to improve the usability and utility of mothur. One of our first experiments was to use mothur to generate standard vector graphic (SVG)-formatted files of heatmaps and Venn diagrams depicting the overlap between microbial communities. Such visuals were helpful for exploring or data; however, I quickly realized that I would never put a mothur-generated figure into a manuscript I wrote. Such visuals require far too much customization to be publication-quality. Although QIIME has incorporated visualization tools through the Emperor package (67), the challenge of users taking default values has downsides as ordinations with black background or publishing 3-D ordinations in a 2-D medium litter the literature. Instead, we have encouraged users to use R packages to visualize mothur-generated results using the minimalR instructional materials that I have developed (http://www.riffomonas.org/minimalR/). A second experiment was the creation of a graphical user interface (GUI) for running mothur. Forcing users to interact with mothur through the command line has been a significant hurdle for many. Unfortunately, the development effort required to create and maintain a GUI is significant and there is limited funding for such efforts. The newest version of QIIME (starting with version 2.0.0) has emphasized interaction with the tools through a GUI (68) and the related QIITA project offers a web-based GUI (69). It remains to be seen how this experiment will go. Another downside of using a GUI is that there is a risk that reproducibility will suffer if users do not have a mechanism to document their mouse clicks. Documentation of commands and parameter values is explicit in mothur as users can provide the software a file with a list of commands and all commands and output are recorded in a logfile. Given the heightened focus on reproducibility in recent

years (70), we have extended significant effort in developing instructional materials teaching users how to organize, document, and execute reproducible pipelines that allow a user to go from raw sequence data to a compiled manuscript with figures through the Riffomonas project (65). A final example of a failed experiment was a collaboration with programmers through Google Summer of Code to develop commands in mothur that ran the random forest and SVM machine learning algorithms. Similar to the challenges of developing attractive visuals, fitting the algorithms' hyperparameters, testing, and deploying the resulting models require a significant amount of customization. Furthermore, machine learning is an active area of research where methods are still being developed and improved. Thankfully, there are numerous R and Python packages that do a better job of developing these models (71, 72). Again, we have put our efforts into developing instructional materials that mothur users can use to fit such models to their data. In each of our "failed" experiments, the real problems were straying from what mothur does well and failing to grasp what we really wanted the innovation to do.

The future. I will continue to develop mothur for as long as other researchers find it useful. One challenge of such a plan is maintaining the funding to support its development. The development of mothur was initially enabled by a subcontract from a Sloan Foundation grant to Mitch Sogin to support his VAMPS (Visualization and Analysis of Microbial Population Structures) initiative. We used that seed funding to secure an NSF grant and then a grant from NIH for tool development as part of their Human Microbiome Project. Since that project expired in 2013, we have not had funding to specifically support mothur's development. I have been fortunate to have start-up and discretionary funds generated from other projects to help support mothur. Although there is funding for new tools, there appears to be little appetite by funders to support existing tools. Emblematic of this was the NIH program, Big Data To Knowledge (BD2K), which solicited proposals through the program announcement "Extended Development, Hardening and Dissemination of Technologies in Biomedical Computing, Informatics and Big Data Science (PA-14-156)". This opportunity appeared perfect, except that the National Institute of Allergy and Infectious Diseases (NIAID), the primary supporter of microbiome research at NIH, did not participate in the announcement. Tools like mothur are clearly successful, but need funding mechanisms to continue to mature and support the needs of the research community.

As with anything in science, methods become passé. When we first developed mothur, T-RFLP and DGGE were still commonly used. Today it would be hard to argue that data from those methods meaningfully advance a study relative to what one could get using 16S rRNA gene sequence data. Looking forward, many want to claim that amplicon sequencing is today's DGGE. They claim that researchers should instead move on to shotgun metagenomic sequencing. It is important to note that the two methods answer fundamentally different questions. 16S rRNA gene sequence data describes the taxonomic composition and metagenomic sequence data tells a researcher about the functional potential and genetic diversity of a community. Both tools provide important information but they cannot easily replace each other. Although metagenomic data does provide highly resolved taxonomic information, the limit of detection is at least an order of magnitude higher than that of amplicon data. For example, we analyzed 10,000 16S rRNA sequences from each of about 500 subjects (73). We can think of this as representing about 1,000,000 genome equivalents (10,000 16S rRNA genes/subject x 500 subjects / 5 16S rRNA gene sequences/genome). Assuming a genome is 4 Mbp, this would represent a sequencing depth of 4 Tbp. Although such a sequencing effort is technically possible, the cost of such an endeavor would be considerable and unlikely to be pursued by most researchers. We estimate that generating and sequencing the libraries at the University of Michigan sequencing core would cost approximately $150 per library. The parallel 16S rRNA gene sequences data would cost approximately $8 per library. Furthermore, analyzing such a large dataset with an approach that captures the full genetic diversity of the community would be financially and technically prohibitive. Going forward, there is still a place for 16S rRNA gene sequencing. Although sequencing technologies will evolve to capture longer and more high quality data, there will likely always be a need for characterizing the taxonomic diversity of microbial communities. With this in mind, there will always be a place for tools like mothur that can analyze amplicon sequence data.

Of course this does not mean that such tools will remain static. We see three key areas that we will continue to help the field to move forward. First, just as we adapted through the transitions from Sanger to 454 to MiSeq and PacBio sequencing platforms (60–62), we must learn whether data from Oxford Nanopore can be an alternative sequencing approach that generates sequence data that is the same quality as existing approaches; thus far, the approach has significant

shortcomings for sequencing 16S rRNA gene sequences (74). As with the earlier platforms, we must better understand its error profile so that sequencing errors can be corrected. We have learned that moving forward requires that we maintain or improve sequence quality. No doubt, datasets and read lengths will improve, but these advances should not be made at the cost of data quality. Second, with these improvements, we will need to continue to improve our algorithms. We have already seen that attempts to use low quality MiSeq and HiSeq data causes computational problems leading to the creation of open and closed reference clustering methods (75, 76). Unfortunately, comparative analyses showed that these methods fail relative to *de novo* clustering methods (57). More work is needed to improve reference-based clustering methods so that larger datasets can be analyzed without sacrificing quality. Finally, there are ongoing controversies that need further exploration. These include the validity and utility of amplicon sequence variants (77), the wisdom of removing low frequency sequences (78), and methods of identifying and removing contaminant 16S rRNA gene sequences (79, 80). With each of these areas of development, the broader community can count on our same data-driven approach to meeting evaluating these questions. It is common for researchers to comment that they pick a specific method or deviate from a suggestion because they "like how the data look". When pressed for an objective definition of how they know the data look "right", they go quiet. Through the use of mock communities and simulations where we actually know what looks right and objective metrics of quality like the MCC or sequencing error rates, we will continue to base recommendations on data rather than a gut feeling.

Conclusion. In the paper announcing mothur, we commented that the relationship between 16S rRNA gene sequencing and analysis is very much like the Red Queen in Lewis Carroll's book, *Through the Looking-Glass*. Although some disagreed with this analogy (81), I still feel it is apt. The sequencing technology and rapacious appetite of researchers continues to race on. At the same time, bioinformatics tools must adapt to facilitate our research. I am confident that mothur will be up to this exciting challenge. Beyond its utility for analyzing amplicon sequence data, mothur's history provides lessons that are helpful for other projects that hope to develop a long historical arc. First, mothur is a product of its time. We have always sought to solve a current need to the best of our ability with the tools we had at the time. There are certainly caveats to any analysis

of 16S rRNA gene sequence data, but if we had waited until those caveats were resolved, the field never would have progressed. Similarly, we made design choices that we probably would not have made had we started the project today. Second, as we have developed mothur, we have attempted to do so in a data-driven approach where we compare multiple methods. It has not merely been enough to propose a new method, we must show that it meaningfully advances the field. Third, through our failures and successes we have learned to focus on what mothur is good at and create products separate from mothur when distinct needs arise. For example, we have learned that mothur should not have a graphical interface or data visualization tools. Instead, we will provide instructional materials to teach users how to use the command line interface and other programs like R for data visualization. Finally, mothur was born out of a need for automating the analysis of large 16S rRNA gene sequence datasets. I realized I had a set of skills to fill that need. It has been refreshing to see the computational skills of the microbial ecology field grow over the past two decades. Looking ahead, we must all take stock of the challenges we face in microbial ecology and how our individual skills and interests can address these challenges to turn them into opportunities.

Acknowledgements

The development of mothur would not be possible without the contributions of its many supporters, developers, and users. Although not a complete list, I would be remiss if I did not express my gratitude to Sarah Westcott and the other members of my research group, Jo Handelsman, Mitch Sogin, Susan Huse, Vincent Young, Lita Proctor, Kendra Mass, and Marcy Balunas for their unique contributions to the continued development of mothur.

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Figure 1. Caption caption caption. Footnotes footnotes footnotes.

- Timeline of mothur/QIIME/etc citations
- Timeline of SRA deposits / 16S/microbiome in PubMed

Figure 2. Caption caption caption. Footnotes footnotes footnotes.

- Screenshot of mothur welcoming page

Figure 3. Caption caption caption. Footnotes footnotes footnotes.

- Screenshot of mothur homepage