OptiFit: a fast method for fitting amplicon sequences to existing OTUs

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Kelly L. Sovacool[1], Sarah L. Westcott[2], M. Brodie Mumphrey[1], Gabrielle A. Dotson[1], Patrick D. Schloss[2]†

1 Department of Computational Medicine and Bioinformatics, University of Michigan, Ann Arbor, MI 48109

2 Department of Microbiology and Immunology, University of Michigan, Ann Arbor, MI 48109

† To whom correspondence should be addressed: pschloss@umich.edu

Abstract

Assigning amplicon sequences to Operational Taxonomic Units (OTUs) is an important step in characterizing the composition of microbial communities across large datasets. OptiClust, a *de novo* OTU clustering method in the mothur program, has been shown to produce higher quality OTU assignments than other methods and at comparable or faster speeds (1, 2). A notable difference between *de novo* clustering and database-dependent methods is that OTU assignments clustered with *de novo* methods are not stable when new sequences are added to a dataset (3). However, in some cases one may wish to incorporate new samples into a previously clustered dataset without performing clustering again on all sequences, such as when deploying a machine learning model where OTUs are features (4). To provide an efficient and robust method to fit amplicon sequence data to existing OTUs, we developed the OptiFit algorithm as a new component of the mothur program.

TODO: summarize results & conclusion

Importance

TODO

Introduction

Amplicon sequencing has become a mainstay of microbial ecology and host-associated microbiome research. Researchers can affordably generate millions of sequences to characterize the composition of hundreds of samples from culture-independent microbial communities. In a typical analysis pipeline, 16S rRNA gene sequences are assigned to Operational Taxonomic Units (OTUs) to facilitate comparison of taxonomic composition between communities. A distance threshold of 3% (or sequence similarity of 97%) is commonly used to cluster sequences into OTUs based on either a reference database or pairwise comparisons of the sequences within the dataset. The method chosen for clustering affects the quality of OTU assignments and thus may impact downstream analyses of community composition (1, 3, 5).

There are three main categories of OTU clustering algorithms: closed reference, open reference, and *de novo* clustering. Closed reference methods assign sequences to a set of pre-made OTUs generated from reference sequences. If a query sequence is not within the distance threshold to any of the reference sequences, it is discarded. While reference-based clustering is generally fast, it is limited by the diversity of the reference database. Rare or novel sequences in the sample will be lost if they are not represented by a similar sequence in the database. *De novo* methods cluster sequences based on their distance to each other, without the use of an external reference. *De novo* clustering overcomes the limitations of reference databases by considering only sequences in the dataset, but is more computationally intensive and generates different OTU assignments when new sequences are introduced. Unstable OTU assignments make it difficult to use *de novo* clustering to compare taxonomic composition of communities between studies or to use machine learning models trained with *de novo* OTUs to make predictions on new data. Open reference methods take a hybrid approach, first performing closed reference clustering, then any sequences that cannot be assigned to reference OTUs are

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clustered *de novo* to create additional OTUs. Previous studies found that the OptiClust *de novo* clustering algorithm created the highest quality OTU assignments of all clustering methods based on the Matthews correlation coefficient (MCC) (1). As a result, we have recommended OptiClust as the preferred method for OTU clustering whenever OTU stability is not required.

- **TODO: current method for open/closed is vsearch against greengenes.**
- **TODO**: 2 categories of clustering: *de novo* and reference based. separate paragraphs. describe opticlust first in de novo paragraph. 2nd paragraph: ref methods are good cause they're fast and don't use much ram. dependent on order of db. people use greengenes, which are rep seqs from 3% otus from full length.
- reader should know what opticlust is, closed & open ref clustering is, strengths & weakness are of each. then we solve these problems.
- **TODO:** note that greengenes is defunct now?!

To overcome the limitations of *de novo* clustering while maintaining OTU quality, we developed OptiFit, a reference-based clustering algorithm in the mothur program which takes existing OTUs as the reference to fit new sequences to. **TODO: more words here?** Here, we tested the OptiFit algorithm with the reference as a database or *de novo* OTUs and compared the performance to existing tools. To evaluate the OptiFit algorithm and compare to existing methods, we used four published datasets isolated from soil (6), marine (7), mouse gut (8), and human gut (9) samples.

Results

The OptiFit algorithm

- **TODO: ask Sarah Westcott to check the accuracy of this description**.
- **TODO: toy example like opticlust with figure**.

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OptiFit leverages the method employed by OptiClust of iteratively assigning sequences to OTUs to produce the highest quality OTUs possible, and extends this method for reference-based clustering. **TODO: more narrative, less documentation-like. TODO: extension of opticlust except ref otus are fixed. just like opticlust only query seqs can move otus.** OptiFit takes as input a list of reference OTUs and their sequences, a list of query sequences to assign to the reference OTUs, the sequence pairs that are within the distance threshold (e.g. 0.03), and the metric to assess clustering quality (default: MCC). Query sequences are randomly seeded in reference OTUs, then for each sequence the algorithm calculates the quality metric based on whether the sequence stays in its current OTU, moves to each of the other OTUs, or is discarded. If two or more OTU assignments are of equal quality, a random number generator is used to break the tie. This process is repeated until the quality metric stabilizes, changing by no more than 0.0001 by default, or until a maximum number of iterations is reached (default: 100). In closed-reference mode, any query sequences that cannot be assigned are thrown out (**TODO: exactly what determines whether a seq can't be assigned?**), and the results only contain OTUs that exist in the original reference. In open-reference mode, unassigned query sequences are clustered *de novo* using OptiClust to generate additional OTUs. The final quality score is reported with the best OTU assignments. There are two strategies for generating OTUs with OptiFit: 1) fit sequences to reference OTUs of an independent database, or 2) split the dataset into a reference and query fraction, cluster the reference sequences *de novo*, then fit the query sequences to the reference OTUs.

TODO: make sure this info is baked into relevant sections, then remove. For each dataset repeated with 100 random seeds, we generated OTUs with OptiFit using both strategies. To compare to existing software, we also clustered OTUs *de novo* using OptiClust and VSEARCH, and with VSEARCH in reference-based mode against the Greengenes database.

Reference clustering with public databases

TODO: use word "map" for what vsearch does, "fit" for what optifit does.

TODO: ref clustering method paragraph here. We assigned blah ref dbs to 3% otus with opticlust. *de novo* clustering the Greengenes database, SILVA non-redundant database, or the Ribosomal Database Project (RDP) (10–12). All clustering was performed at a distance threshold of 0.03 and OTU quality was evaluated using the MCC as described previously (1). To evaluate reference-based clustering with independent databases, we fit each dataset to reference OTUs generated by repeated 100 times.

In open reference mode, fitting the datasets to reference OTUs with OptiFit produced OTUs of similar quality (1.01% difference in median MCC) as clustering the datasets *de novo* with OptiClust across all datasets and reference databases. OptiFit produced higher quality OTUs than VSEARCH when open reference clustering against the Greengenes database, with median MCC scores of 0.82 and 0.52 (respectively). However, OptiClust and VSEARCH each ran 28.39% and 181.05% faster than OptiFit in open reference mode. *De novo* clustering with OptiClust produced 56.08% higher quality OTUs than VSEARCH, but performed only 48.79% as fast as VSEARCH.

In closed reference mode against databases, OptiFit produced lower quality OTUs than OptiClust by 19.25 on average. Fitting sequences to Greengenes and SILVA in closed reference mode performed similarly with median MCC scores of 0.80 and 0.72 respectively, while when fitting to RDP the median MCC dropped to 0.33. An average of only 0.31 of query sequences were fit to reference database OTUs in closed reference mode across all dataset/database combinations. VSEARCH was able to map 41.83 more query sequences than OptiFit to the Greengenes reference database. In terms of run time, closed reference OptiFit outperformed OptiClust by -28.92 and VSEARCH by -77.75.

Reference clustering with split datasets

A split dataset strategy was employed to assess how well OptiFit performs for tasks where new sequences are added to existing OTUs, such as when comparing OTUs across studies or making predictions with machine learning models. Datasets were randomly split into a reference fraction and a query fraction. Reference sizes from 10% to 80% of the sequences were created, with the remaining sequences used for the query. Reference sequences were clustered *de novo* with OptiClust, then query sequences were fit to the *de novo* OTUs with OptiFit.

OTU quality from the split dataset strategy with OptiFit was highly similar to that from *de novo* clustering the whole dataset with OptiClust regardless of mode (**TODO: diff in MCC medians**). OTU quality was remarkably stable across 100 different random seeds (**TODO: coeff of variation?**). In terms of runtime, closed reference OptiFit performed faster than OptiClust on whole datasets by **Z%**. In open reference mode, OptiClust performed **X to Y%** faster than OptiFit only when the OptiFit reference fraction was 30% or less. The split dataset strategy performed just as well as the database strategy in open reference mode regardless of database used, and outperformed the database strategy in closed reference mode by **W%**.

We also tested three methods for selecting the fraction of sequences to be used as the reference; a simple random sample, weighting sequences by relative abundance, and weighting by similarity to other sequences in the dataset. OTU quality was similar with the simple and abundance-weighted sampling (median MCCs **X** and **Y** respectively), but **Z%** worse with similarity-weighted sampling. In closed reference mode, the fraction of query sequences that can be fit to the reference OTUs decreases as the reference fraction increases from an MCC of **A** with **J** reference sequences to an MCC of **B** with **K** reference sequences.

Discussion

We developed a new algorithm for fitting sequences to existing OTUs and have demonstrated its suitability for reference-based clustering. OptiFit makes the iterative method employed by OptiClust available for tasks where reference-based clustering is required. We have shown that OTU quality is similar between OptiClust and OptiFit in open reference mode, regardless of strategy employed. Open reference OptiFit performs slower than OptiClust due to the additional *de novo* clustering step, so users may prefer OptiClust for tasks that do not require reference OTUs.

When fitting to public databases, OTU quality dropped in closed reference mode to different degrees depending on the database and dataset source, and no more than half of query sequences were able to be fit to OTUs across any dataset/database combination. This may reflect limitations of reference databases, which are unlikely to contain sequences from rare and novel microbes. This drop in quality was most notable with RDP, which contains only about 21,000 sequences compared to over 200,000 sequences in SILVA and Greengenes each at the time of this writing. We recommend that users who require an independent reference database opt for large databases with good coverage of microbial diversity. Since OptiClust performs faster than open reference OptiFit and creates higher quality OTUs than closed reference OptiFit with the database strategy, we recommend using OptiClust rather than fitting to a database whenever stable OTUs are not required for the study at hand.

The OptiClust and OptiFit algorithms provided by mothur produced higher quality OTUs than VSEARCH in open reference, closed reference, or *de novo* modes. However, VSEARCH was able to fit more sequence into OTUs than OptiFit in closed reference mode. While both mothur and VSEARCH use a distance or similarity threshold for determining how to assign sequences to OTUs, VSEARCH is more permissive than mothur. The OptiFit and OptiClust algorithms use all of the sequences to define an OTU, requiring that all

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pairs of sequences (including reference and query sequences) in an OTU are within the distance threshold without penalizing the MCC. In contrast, VSEARCH only requires each query sequence to be similar to the single sequence that seeded the OTU. In this way, VSEARCH sacrifices OTU quality in order to allow more sequences to fit to OTUs. Users who require closed reference clustering to the Greengenes database may prefer to use VSEARCH if they wish to maximize the fraction of sequences that can be fit at the cost of OTU quality. However, mothur's OptiClust or OptiFit are recommended for *de novo* or open reference clustering to produce OTUs of the highest possible quality.

When fitting with the split dataset strategy, OTU quality was remarkably similar when reference sequences were selected by a simple random sample or weighted by abundance, but quality was slightly worse when sequences were weighted by similarity. We recommend using a simple random sample since the more sophisticated reference selection methods do not offer any benefit. The similarity in OTU quality between OptiClust and OptiFit with this strategy demonstrates the suitability of using OptiFit to fit sequences to existing OTUs, such as when using already-trained machine learning models to make predictions on new data or comparing OTUs across studies. However, when stable OTUs are not required, we recommend using OptiClust for *de novo* clustering over the split strategy with OptiFit since OptiClust is simpler to execute but performs similarly in terms of both run time and OTU quality.

TODO: big picture concluding paragraph. We have developed a new clustering algorithm that allows users to produce high quality OTUs using already existing OTUs as a reference. **TODO: Point to courtney's paper metaphorically. wow what a cool application someone should do *wink wink*.**

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Materials and Methods

Data Processing Steps

We downloaded 16S rRNA gene amplicon sequences from four published datasets isolated from soil (6), marine (7), mouse gut (8), and human gut (9) samples. Raw sequences were processed using mothur according to the Schloss Lab MiSeq SOP as described in the mothur wiki and accompanying study by Kozich *et al.* (13, 14). These steps included trimming and filtering for quality, aligning to the SILVA reference alignment (11), discarding sequences that aligned outside the V4 region, removing chimeric reads with UCHIME (15), and calculating distances between all pairs of sequences within each dataset prior to clustering.

Reference database clustering

To generate reference OTUs from independent databases, we downloaded sequences from the Greengenes database (v13_8_99) (10), SILVA non-redundant database (v132) (11), and the Ribosomal Database Project (v16) (12). These sequences were processed using the same steps outlined above followed by clustering sequences into *de novo* OTUs with OptiClust. Processed reads from each of the four datasets were clustered with OptiFit to the reference OTUs generated from each of the three databases. When reference clustering with VSEARCH, processed datasets were fit directly to the unprocessed Greengenes reference alignment, since this method is how VSEARCH is typically used by the QIIME2 software reference-based clustering (16, 17).

Split dataset clustering

For each dataset, a fraction of the sequences was selected to be clustered *de novo* into reference OTUs with OptiClust. We used three methods for selecting the fraction of sequences to be used as the reference; a simple random sample, weighting sequences by

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relative abundance, and weighting by similarity to other sequences in the dataset. Dataset splitting was repeated with reference fractions ranging from 10% to 80% of the dataset and for 100 random seeds. For each dataset split, the remaining sequences were assigned to the reference OTUs with OptiFit.

Benchmarking

Since OptiClust and OptiFit employ a random number generator to break ties when OTU assignments are of equal quality, they produce slightly different OTU assignments when repeated with different random seeds. To capture any variation in OTU quality or execution time, clustering was repeated with 100 random seeds for each combination of parameters and input datasets. We used the benchmark feature provided by Snakemake to measure the run time of every clustering job. We calculated the MCC on each set of OTUs to quantify the quality of clustering, as described by Westcott *et al.* (1).

Data and Code Availability

We implemented the analysis workflow in Snakemake (18) and wrote scripts in R (19), Python (20), and GNU bash (21). Software used includes mothur v1.45.0 (2), VSEARCH v2.13.3 (22), numpy (23), the Tidyverse metapackage (24), R Markdown (25), the SRA toolkit (26), and the conda environment manager (27). The complete workflow, manuscript, and conda environment are available at **TODO: UPDATED REPO LINK**.

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to submit the work for publication.

Author Contributions

KLS wrote the analysis code, evaluated the algorithm, and wrote the original draft of the manuscript. SLW designed and implemented the OptiFit algorithm and assisted in debugging the analysis code. MBM and GAD contributed analysis code. PDS conceived the study, supervised the project, and assisted in debugging the analysis code. All authors reviewed and edited the manuscript.

1. **Westcott SL**, **Schloss PD**. 2017. OptiClust, an Improved Method for Assigning Amplicon-Based Sequence Data to Operational Taxonomic Units. mSphere **2**:e00073–17. doi:10.1128/mSphereDirect.00073-17.

2. **Schloss PD**, **Westcott SL**, **Ryabin T**, **Hall JR**, **Hartmann M**, **Hollister EB**, **Lesniewski RA**, **Oakley BB**, **Parks DH**, **Robinson CJ**, **Sahl JW**, **Stres B**, **Thallinger GG**, **Van Horn DJ**, **Weber CF**. 2009. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology **75**:7537–7541. doi:10.1128/AEM.01541-09.

3. **Westcott SL**, **Schloss PD**. 2015. De novo clustering methods outperform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units. PeerJ **3**:e1487. doi:10.7717/peerj.1487.

4. **Topçuoğlu BD**, **Lesniak NA**, **Ruffin M**, **Wiens J**, **Schloss PD**. 2019. Effective application of machine learning to microbiome-based classification problems. bioRxiv 816090. doi:10.1101/816090.

5. **Schloss PD**. 2016. Application of a Database-Independent Approach To Assess the Quality of Operational Taxonomic Unit Picking Methods. mSystems **1**:e00027–16.

260 doi:10.1128/mSystems.00027-16.

261 6. **Johnston ER**, **Rodriguez-R LM**, **Luo C**, **Yuan MM**, **Wu L**, **He Z**, **Schuur EAG**, **Luo Y**,

262 **Tiedje JM**, **Zhou J**, **Konstantinidis KT**. 2016. Metagenomics Reveals Pervasive Bacterial

263 Populations and Reduced Community Diversity across the Alaska Tundra Ecosystem. Front

264 Microbiol **7**. doi:10.3389/fmicb.2016.00579.

265 7. **Henson MW**, **Pitre DM**, **Weckhorst JL**, **Lanclos VC**, **Webber AT**, **Thrash JC**. 2016.

266 Artificial Seawater Media Facilitate Cultivating Members of the Microbial Majority from the

267 Gulf of Mexico. mSphere **1**. doi:10.1128/mSphere.00028-16.

268 8. **Schloss PD**, **Schubert AM**, **Zackular JP**, **Iverson KD**, **Young VB**, **Petrosino JF**. 2012.

269 Stabilization of the murine gut microbiome following weaning. Gut Microbes **3**:383–393.

270 doi:10.4161/gmic.21008.

271 9. **Baxter NT**, **Ruffin MT**, **Rogers MAM**, **Schloss PD**. 2016. Microbiota-based model

272 improves the sensitivity of fecal immunochemical test for detecting colonic lesions. Genome

273 Med **8**:37. doi:10.1186/s13073-016-0290-3.

274 10. **DeSantis TZ**, **Hugenholtz P**, **Larsen N**, **Rojas M**, **Brodie EL**, **Keller K**, **Huber**

275 **T**, **Dalevi D**, **Hu P**, **Andersen GL**. 2006. Greengenes, a Chimera-Checked 16S

276 rRNA Gene Database and Workbench Compatible with ARB. AEM **72**:5069–5072.

277 doi:10.1128/AEM.03006-05.

278 11. **Quast C**, **Pruesse E**, **Yilmaz P**, **Gerken J**, **Schweer T**, **Yarza P**, **Peplies J**, **Glöckner**

279 **FO**. 2013. The SILVA ribosomal RNA gene database project: Improved data processing

280 and web-based tools. Nucleic Acids Research **41**:D590–D596. doi:10.1093/nar/gks1219.

281 12. **Cole JR**, **Wang Q**, **Fish JA**, **Chai B**, **McGarrell DM**, **Sun Y**, **Brown CT**, **Porras-Alfaro**

282 **A**, **Kuske CR**, **Tiedje JM**. 2014. Ribosomal Database Project: Data and tools for high

283 throughput rRNA analysis. Nucl Acids Res **42**:D633–D642. doi:10.1093/nar/gkt1244.

13. **Schloss PD**, **Westcott SL**. MiSeq SOP. https://mothur.org/MiSeq_SOP.

14. **Kozich JJ**, **Westcott SL**, **Baxter NT**, **Highlander SK**, **Schloss PD**. 2013. Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. Appl Environ Microbiol **79**:5112–5120. doi:10.1128/AEM.01043-13.

15. **Edgar RC**, **Haas BJ**, **Clemente JC**, **Quince C**, **Knight R**. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics **27**:2194–2200. doi:10.1093/bioinformatics/btr381.

16. **Bolyen E**, **Rideout JR**, **Dillon MR**, **Bokulich NA**, **Abnet CC**, **Al-Ghalith GA**, **Alexander H**, **Alm EJ**, **Arumugam M**, **Asnicar F**, **Bai Y**, **Bisanz JE**, **Bittinger K**, **Brejnrod A**, **Brislawn CJ**, **Brown CT**, **Callahan BJ**, **Caraballo-Rodríguez AM**, **Chase J**, **Cope EK**, **Da Silva R**, **Diener C**, **Dorrestein PC**, **Douglas GM**, **Durall DM**, **Duvallet C**, **Edwardson CF**, **Ernst M**, **Estaki M**, **Fouquier J**, **Gauglitz JM**, **Gibbons SM**, **Gibson DL**, **Gonzalez A**, **Gorlick K**, **Guo J**, **Hillmann B**, **Holmes S**, **Holste H**, **Huttenhower C**, **Huttley GA**, **Janssen S**, **Jarmusch AK**, **Jiang L**, **Kaehler BD**, **Kang KB**, **Keefe CR**, **Keim P**, **Kelley ST**, **Knights D**, **Koester I**, **Kosciolek T**, **Kreps J**, **Langille MGI**, **Lee J**, **Ley R**, **Liu Y-X**, **Loftfield E**, **Lozupone C**, **Maher M**, **Marotz C**, **Martin BD**, **McDonald D**, **McIver LJ**, **Melnik AV**, **Metcalf JL**, **Morgan SC**, **Morton JT**, **Naimey AT**, **Navas-Molina JA**, **Nothias LF**, **Orchanian SB**, **Pearson T**, **Peoples SL**, **Petras D**, **Preuss ML**, **Pruesse E**, **Rasmussen LB**, **Rivers A**, **Robeson MS**, **Rosenthal P**, **Segata N**, **Shaffer M**, **Shiffer A**, **Sinha R**, **Song SJ**, **Spear JR**, **Swafford AD**, **Thompson LR**, **Torres PJ**, **Trinh P**, **Tripathi A**, **Turnbaugh PJ**, **Ul-Hasan S**, **van der Hooft JJJ**, **Vargas F**, **Vázquez-Baeza Y**, **Vogtmann E**, **von Hippel M**, **Walters W**, **Wan Y**, **Wang M**, **Warren J**, **Weber KC**, **Williamson CHD**, **Willis AD**, **Xu ZZ**, **Zaneveld JR**, **Zhang Y**, **Zhu Q**, **Knight R**, **Caporaso JG**. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol **37**:852–857. doi:10.1038/s41587-019-0209-9.

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17. Clustering sequences into OTUs using q2-vsearch QIIME 2 2021.2.0 documentation. https://docs.qiime2.org/2021.2/tutorials/otu-clustering/.

18. **Köster J**, **Rahmann S**. 2012. Snakemake a scalable bioinformatics workflow engine. Bioinformatics **28**:2520–2522. doi:10.1093/bioinformatics/bts480.

19. **R Core Team**. 2020. R: A language and environment for statistical computing. Manual, R Foundation for Statistical Computing, Vienna, Austria.

20. **Van Rossum G**, **Drake FL**. 2009. Python 3 Reference Manual | Guide books.

21. Bash Reference Manual. https://www.gnu.org/software/bash/manual/bash.html.

22. **Rognes T**, **Flouri T**, **Nichols B**, **Quince C**, **Mahé F**. 2016. VSEARCH: A versatile open source tool for metagenomics. PeerJ **4**:e2584. doi:10.7717/peerj.2584.

23. **Harris CR**, **Millman KJ**, **van der Walt SJ**, **Gommers R**, **Virtanen P**, **Cournapeau D**, **Wieser E**, **Taylor J**, **Berg S**, **Smith NJ**, **Kern R**, **Picus M**, **Hoyer S**, **van Kerkwijk MH**, **Brett M**, **Haldane A**, **del Río JF**, **Wiebe M**, **Peterson P**, **Gérard-Marchant P**, **Sheppard K**, **Reddy T**, **Weckesser W**, **Abbasi H**, **Gohlke C**, **Oliphant TE**. 2020. Array programming with NumPy. Nature **585**:357–362. doi:10.1038/s41586-020-2649-2.

24. **Wickham H**, **Averick M**, **Bryan J**, **Chang W**, **McGowan LD**, **François R**, **Grolemund G**, **Hayes A**, **Henry L**, **Hester J**, **Kuhn M**, **Pedersen TL**, **Miller E**, **Bache SM**, **Müller K**, **Ooms J**, **Robinson D**, **Seidel DP**, **Spinu V**, **Takahashi K**, **Vaughan D**, **Wilke C**, **Woo K**, **Yutani H**. 2019. Welcome to the Tidyverse. Journal of Open Source Software **4**:1686. doi:10.21105/joss.01686.

25. **Xie Y**, **Allaire JJ**, **Grolemund G**. 2018. R Markdown: The Definitive Guide. Taylor & Francis, CRC Press.

26. SRA-Tools - NCBI. http://ncbi.github.io/sra-tools/.

333 27. 2016. Anaconda Software Distribution. Anaconda Documentation. Anaconda Inc.

334 **References**