GeFaST: An improved method for OTU assignment by generalising Swarm's fastidious clustering
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Additional file 1: Supplementary information on data and analyses

1 Information on mock-community data

The mock-community data sets `uneven` and `even` have been borrowed from the clustering analysis of the original Swarm paper [1]. They comprise genome isolates from 49 bacterial and 10 archaeal strains. Mahé et al. collated two strains of *Methanococcus maripaludis* and two strains of *Shewanella baltica* because they did not use taxonomic classifications beyond the species level. The 47 bacterial species belong to 17 phyla and 22 classes, while the 10 archaeal species cover three phyla and five classes. More details on the biological composition are provided in Supplement Table 1. Information on the sequencing workflow are available in the original Swarm paper [1].

The ground truth for this analysis was established by matching the sequences against a 16S reference data set. This data set was hand-picked from the GreenGenes database [2] based on the list of organisms in the mock community (Mahé et al., personal communication). Using a minimum sequence identity of, e.g., 97 %, the matching was performed through VSEARCH ([3], v2.7.1) with the `usearch_global` option and picking the closest hit.

The mock-community data sets (`even`, `uneven`) and the `references` are available online.

2 Evaluation on mock-community data

In order to evaluate the clustering quality of GeFaST, we first performed an evaluation very similar to the one presented in the original Swarm paper [1]. However, we included some additional and newer versions of the compared tools in our evaluation.
As described in the main article, the clustering quality was assessed via a comparison of the OTUs obtained from the clustering tools with a ground truth. This ground truth was determined as described in the previous section. The results shown in the main article are based on the common 97 % threshold targeting a distinction at the species level. The following subsections present the results of the evaluation with different similarity thresholds for more and less fine-grained OTU calling.

2.1 95 % ground truth

Repetition of the mock-community analysis with a ground truth determined using a minimum sequence identity of 95 % (Supplement Figure 1). 87.5 % of the sequences in `uneven` and 74.2 % of the ones in `even` matched against the reference.

2.2 99 % ground truth

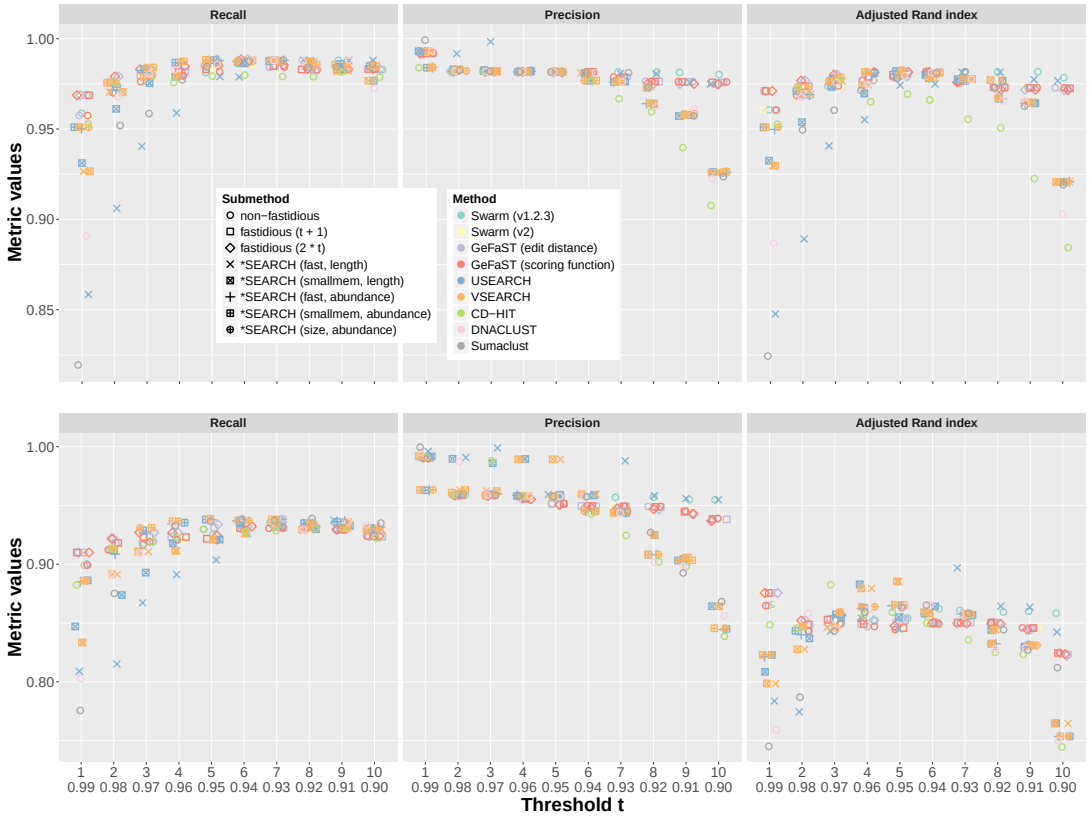
Repetition of the mock-community analysis with a ground truth determined using a minimum sequence identity of 99 % (Supplement Figure 2). 53.0 % of the sequences in `uneven` and 36.6 % of the ones in `even` matched against the reference.

2.3 Subsampling

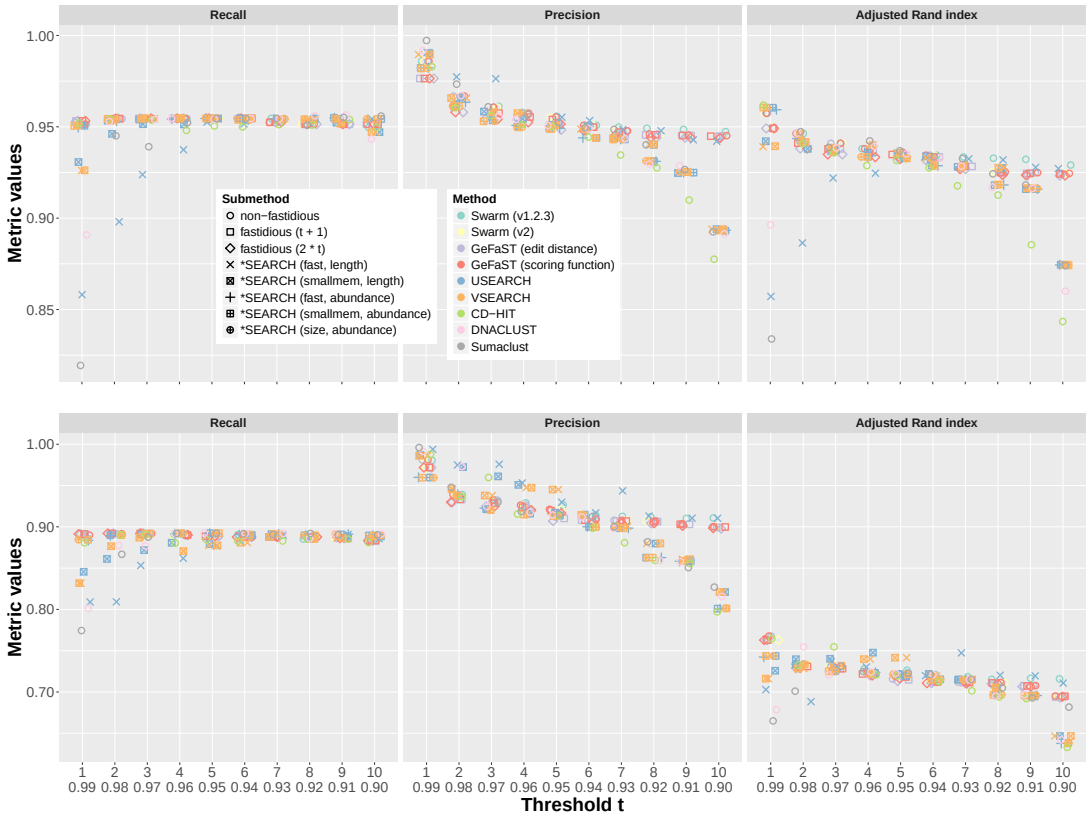
Similarly to `eldermet`, we also performed a clustering-quality analysis on random subsamples of `uneven` (Supplement Figure 3) and `even` (Supplement Figure 4). Again, we subsampled each of the two data sets five times at a level of 80 % and clustered each subsample with all tools for the different thresholds. The thresholds and metrics were the same as for `eldermet`. In contrast to the `eldermet` analysis, we did not need to reduce the data set and determined the ground truths using a 97 % minimum sequence identity on the mock-community reference data set.

	Phylum	Class	Species / genome name
Bacteria	Acidobacteria	Acidobacteriae	Acidobacterium capsulatum ATCC 51196
	Actinobacteria	Actinobacteria	Salinispora arenicola CNS-205
			Salinispora tropica CNB-440
	Aquificae	Aquificae	Hydrogenobaculum sp. Y04AAS1
			Persephonella marina EX-H1
			Sulfurihydrogenibium sp. YO3AOP1
			Sulfurihydrogenibium yellowstonense SS-5
	Bacteroidetes	Bacteroidia	Bacteroides thetaiotaomicron VPI-5482
			Bacteroides vulgatus ATCC 8482
			Porphyromonas gingivalis ATCC 33277
	Chlorobi	Chlorobia	Chlorobaculum tepidum TLS
			Chlorobium limicola DSM 245
			Chlorobium phaeobacteroides DSM 266
			Chlorobium phaeovibrioides DSM 265
			Pelodictyon phaeoclathratiforme BU-1
	Chloroflexi	Chloroflexi	Chloroflexus aurantiacus J-10-fi
			Herpetosiphon aurantiacus ATCC 23779
	Cyanobacteria	unclassified	Nostoc sp. PCC 7120
	Dictyoglomi	Dictyoglomia	Dictyoglomus turgidum DSM 6724
	Firmicutes	Bacilli	Enterococcus faecalis V583
		Clostridia	Anaerocellum thermophilum Z-1320, DSM 6725
			Caldicellulosiruptor saccharolyticus DSM 8903
			Clostridium thermocellum ATCC 27405
			Thermoanaerobacter pseudethanolicus ATCC 33223
	Fusobacteria	Fusobacteria	Fusobacterium nucleatum ATCC 25586
	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonas aurantiaca T-27T
	Planctomycetes	Planctomycetacia	Rhodopirellula baltica SH 1
	Proteobacteria	Alphaproteobacteria	Rhodospirillum rubrum ATCC 11170
			Ruegeria pomeroyi DSS-3
			Sulfitobacter sp. EE-36
			Sulfitobacter sp. NAS-14.1
			Zymomonas mobilis ZM4
		Betaproteobacteria	Bordetella bronchiseptica RB50
			Burkholderia xenovorans LB400
			Leptothrix cholodnii SP-6
			Nitrosomonas europaea ATCC 19718
		Gammaproteobacteria	Shewanella baltica OS185
			Shewanella baltica OS223
		Deltaproteobacteria	Desulfovibrio desulfuricans ATCC 27774
			Desulfovibrio piger ATCC 29098
	Spirochaetes	Spirochaetes	Treponema denticola ATCC 35405
	Thermi	Deinococci	Deinococcus radiodurans R1
		Thermi	Thermus thermophilus HB8
	Thermotogae	Thermotogae	Thermotoga neapolitana DSM 4359
			Thermotoga petrophila RKU-1
			Thermotoga sp. RQ2
	Verrucomicrobia	Verrucomicrobiae	Akkermansia muciniphila ATCC BAA-835
Archaea	Crenarchaeota	Thermoprotei	Ignicoccus hospitalis KIN4/I
			Pyrobaculum aerophilum IM2
			Pyrobaculum calidifontis JCM 11548
			Sulfolobus tokodaii 7(S311)
	Euryarchaeota	Archaeoglobi	Archaeoglobus fulgidus DSM 4304
		Methanococci	Methanocaldococcus jannaschii DSM 2661
			Methanococcus maripaludis C5
			Methanococcus maripaludis S2
		Thermococci	Pyrococcus horikoshii OT3
	Nanoarchaeota	Nanoarchaea	Nanoarchaeum equitans Kin4-M

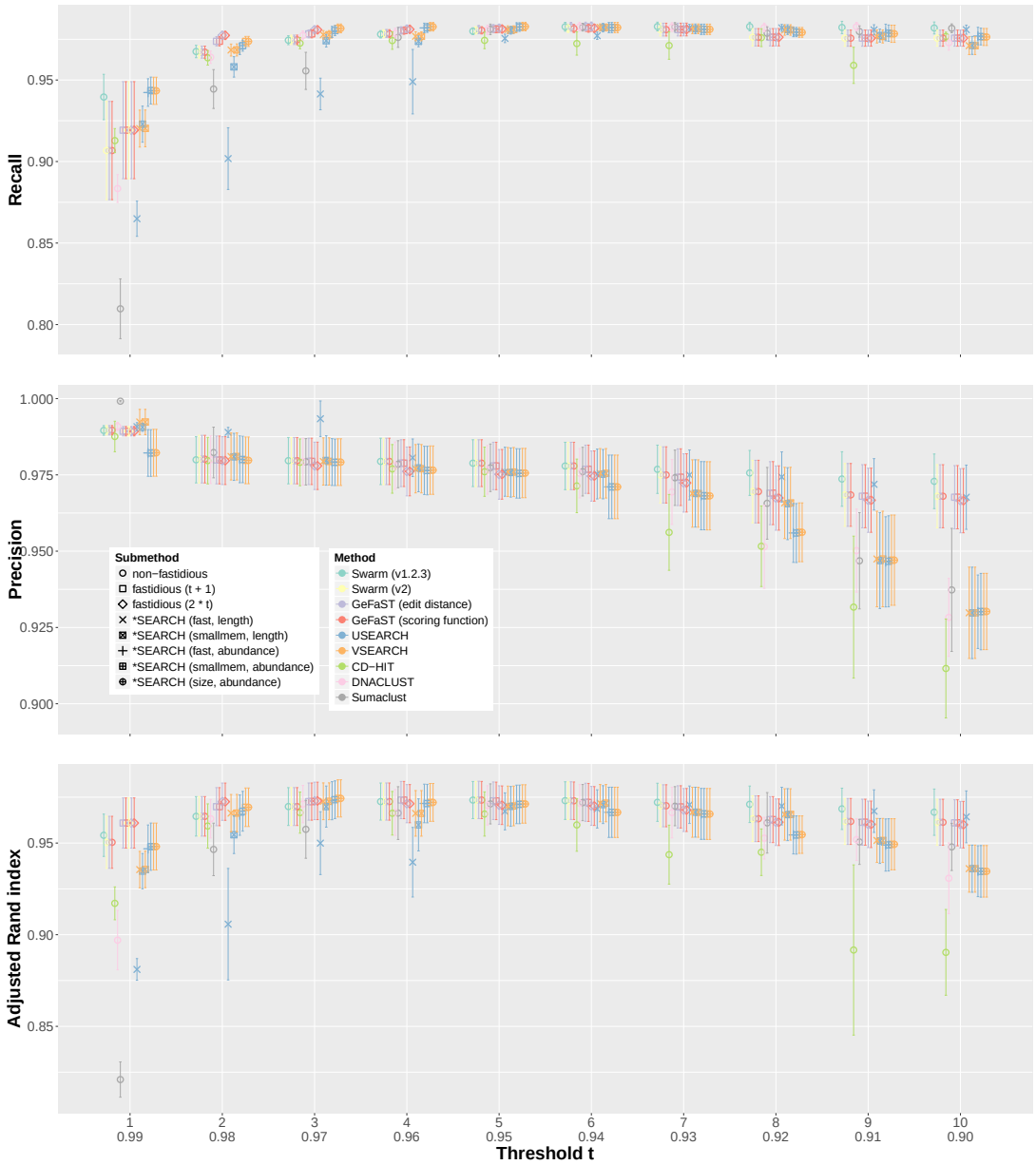
Supplement Table 1: Biological composition of the mock communities. Adapted from [1, Suppl. Tab. 1].



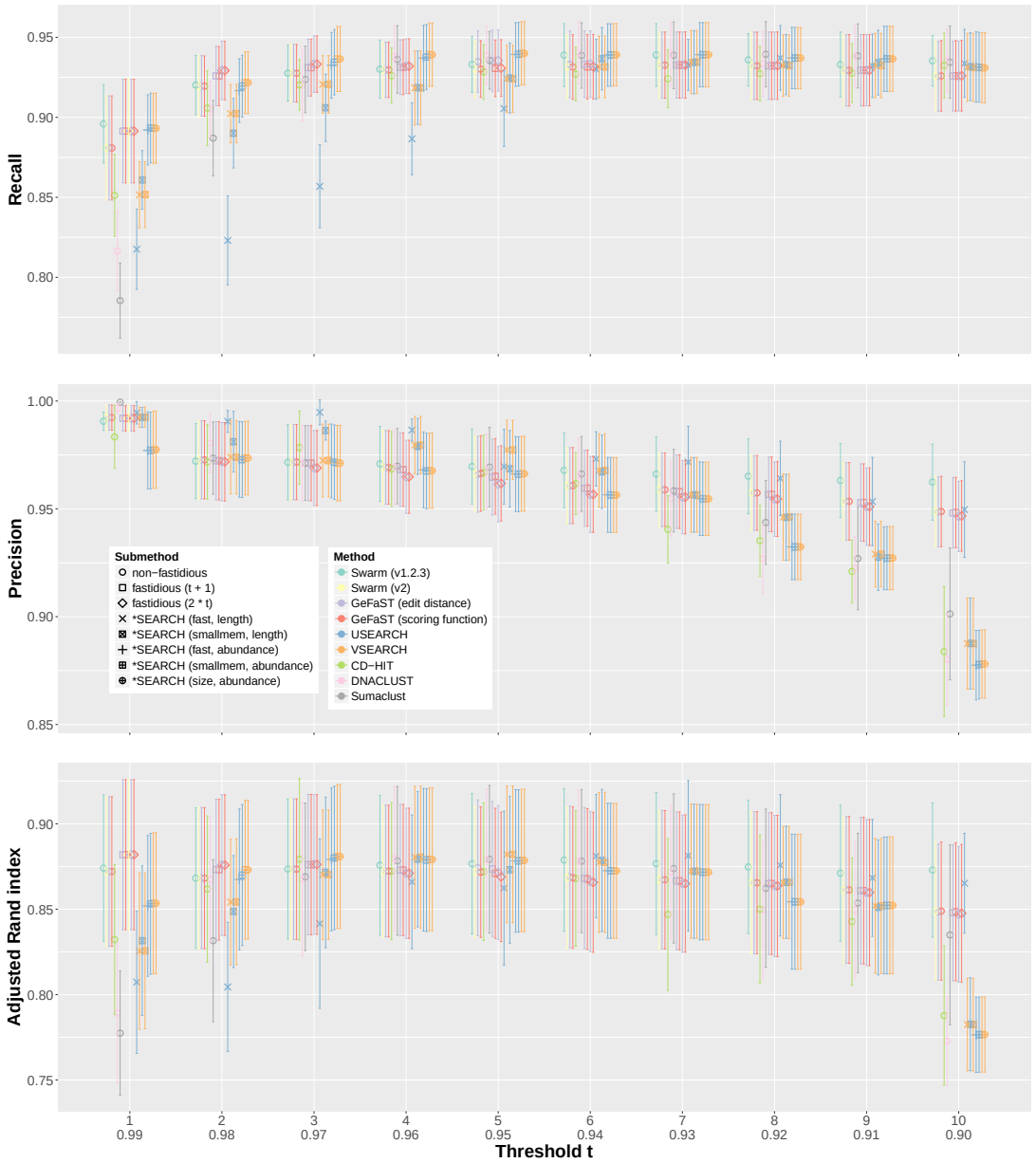
Supplement Figure 1: Comparison of clustering quality on `uneven` (*top*) resp. `even` (*bottom*) mock-community data set for 10 different thresholds using a 95 % ground truth.



Supplement Figure 2: Comparison of clustering quality on `uneven` (*top*) resp. `even` (*bottom*) mock-community data set for 10 different thresholds using a 99 % ground truth.



Supplement Figure 3: Comparison of clustering quality on the `uneven` data set for 10 different thresholds. The average values are determined from five random subsamples (each comprising 80 % of the data set). The standard deviation is indicated by the error bars.



Supplement Figure 4: Comparison of clustering quality on the `even` data set for 10 different thresholds. The average values are determined from five random subsamples (each comprising 80 % of the data set). The standard deviation is indicated by the error bars.

3 Evaluation on natural data

In order to complement the analysis on mock-community data, we also performed an evaluation on the natural `eldermet` data set. Since establishing a ground truth on natural data is harder, the analysis was preceded by a preprocessing of the data set. While de novo clustering assigns all sequences to clusters, closed-reference clustering discards those sequences that cannot be assigned to a reference. Hence, a ground truth resulting from closed-reference clustering might cover only a small proportion of the sequences in the de novo clusters. As this can heavily skew the clustering metrics, we applied the following steps to address this issue:

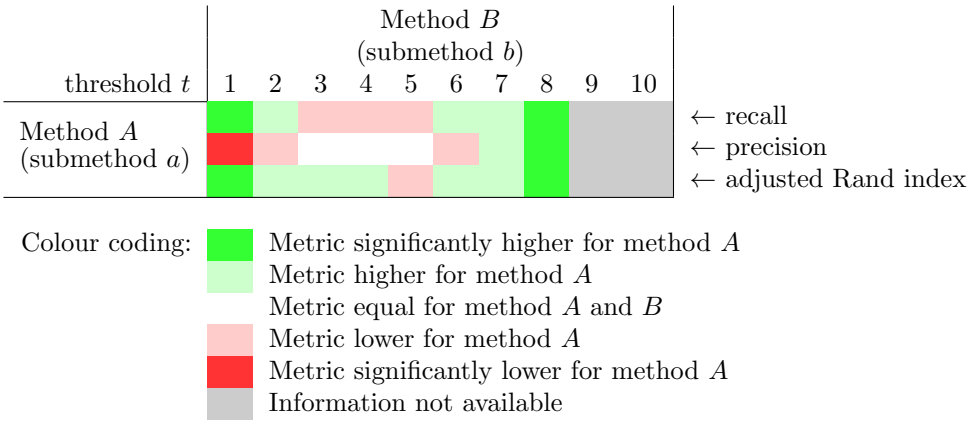
1. Match the dereplicated `eldermet` data set against the 97 % representative set of the SILVA database ([4], release 128).
2. Replace the identifiers of SILVA representatives in the resulting assignment with their actual taxonomic information.
3. Remove the species-level information in the taxonomic assignment (if existent).
4. Discard entries where genus information is missing or ambiguous.
5. Reduce `eldermet` to those sequences remaining in taxonomic assignment.

The closed-reference clustering of step 1 was conducted with VSEARCH (v2.7.1) and a minimum sequence identity of 95 %. The reduced `eldermet` data set and taxonomic assignment were the inputs for the subsequent clustering-quality evaluation.

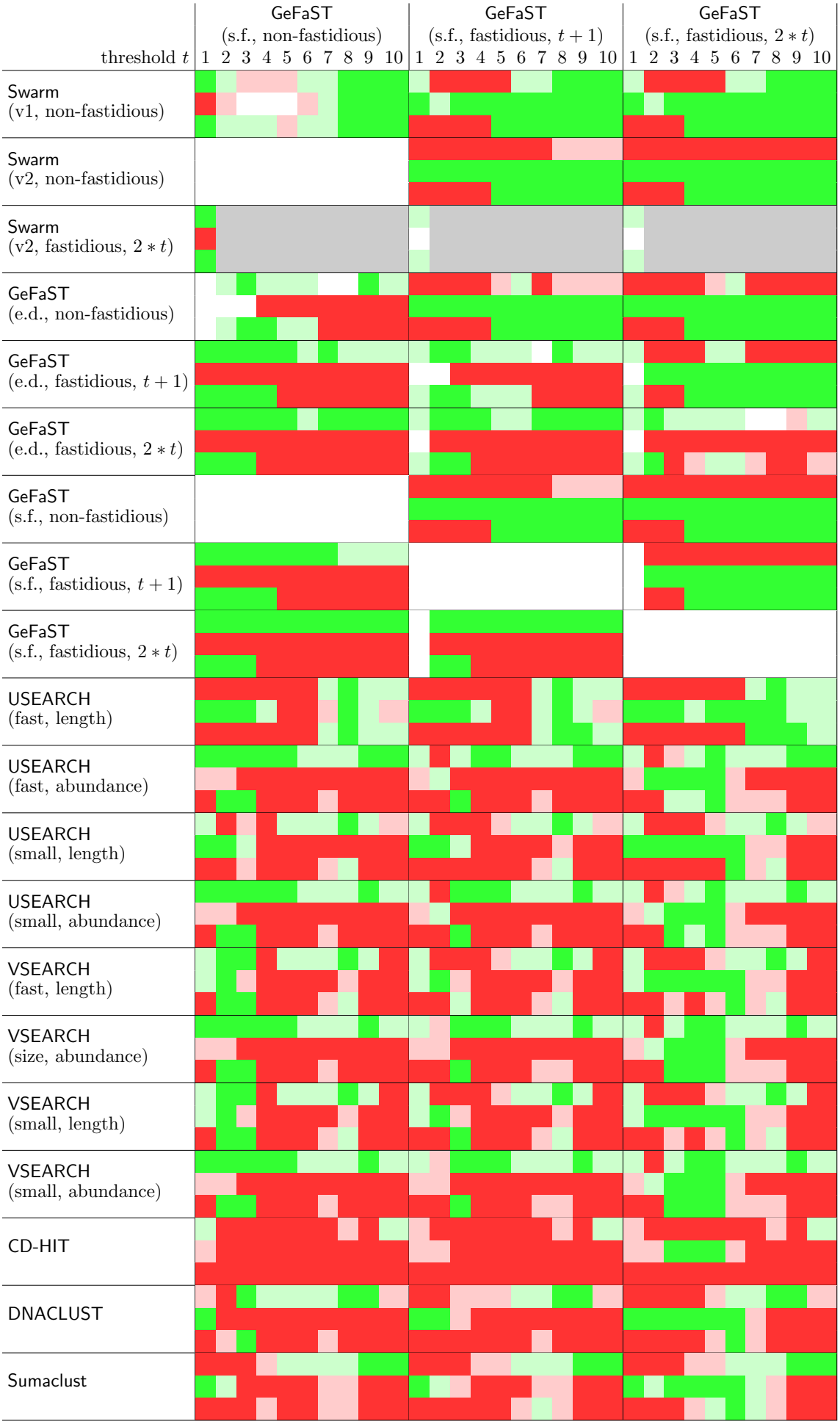
4 Significance of clustering-quality results

We assessed the significance of the differences in clustering quality between the different tools. To this end, we used the results of the mock-community evaluations in Section 2.3 and of the quality analysis on `eldermet` from the main article.

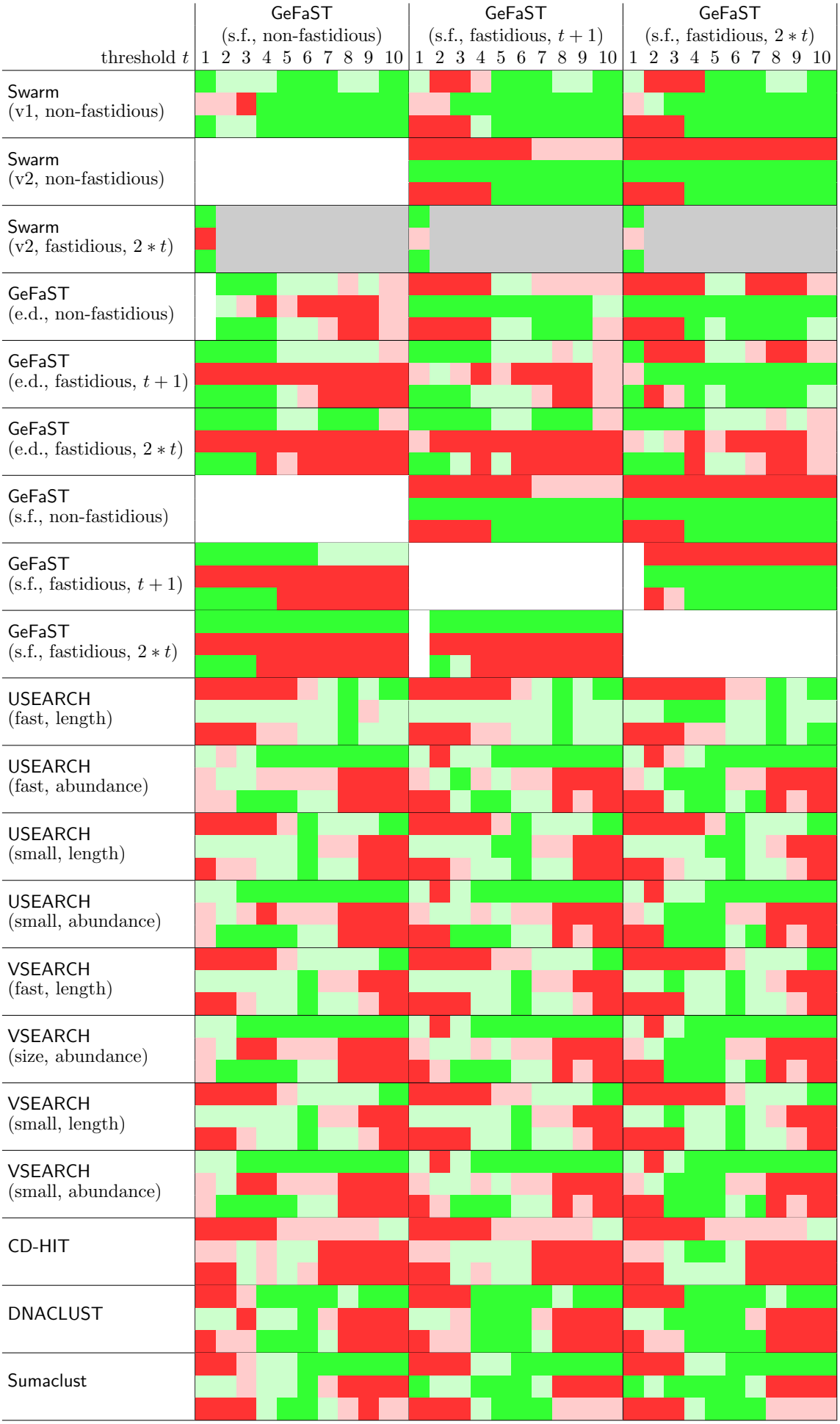
Here, we present an evaluation of the statistical significance of the differences between the tested tools and GeFaST (in scoring-function mode) through paired two-sided t-tests with a significance level of 0.05. Two methods (with certain submethods, if applicable) were compared over all examined thresholds for a set of subsamples. One t-test used the measurements of two methods for a specific combination of data set, metric and threshold. When only Swarm and GeFaST were involved, we used the same threshold for both methods. In a comparison between a method using a global threshold (e.g. VSEARCH) and GeFaST, we used a given local clustering threshold t for GeFaST and $t' = 1 - t/100$ as the global threshold for the other method. The statistical significance is depicted in one table per data set as shown in the example below:



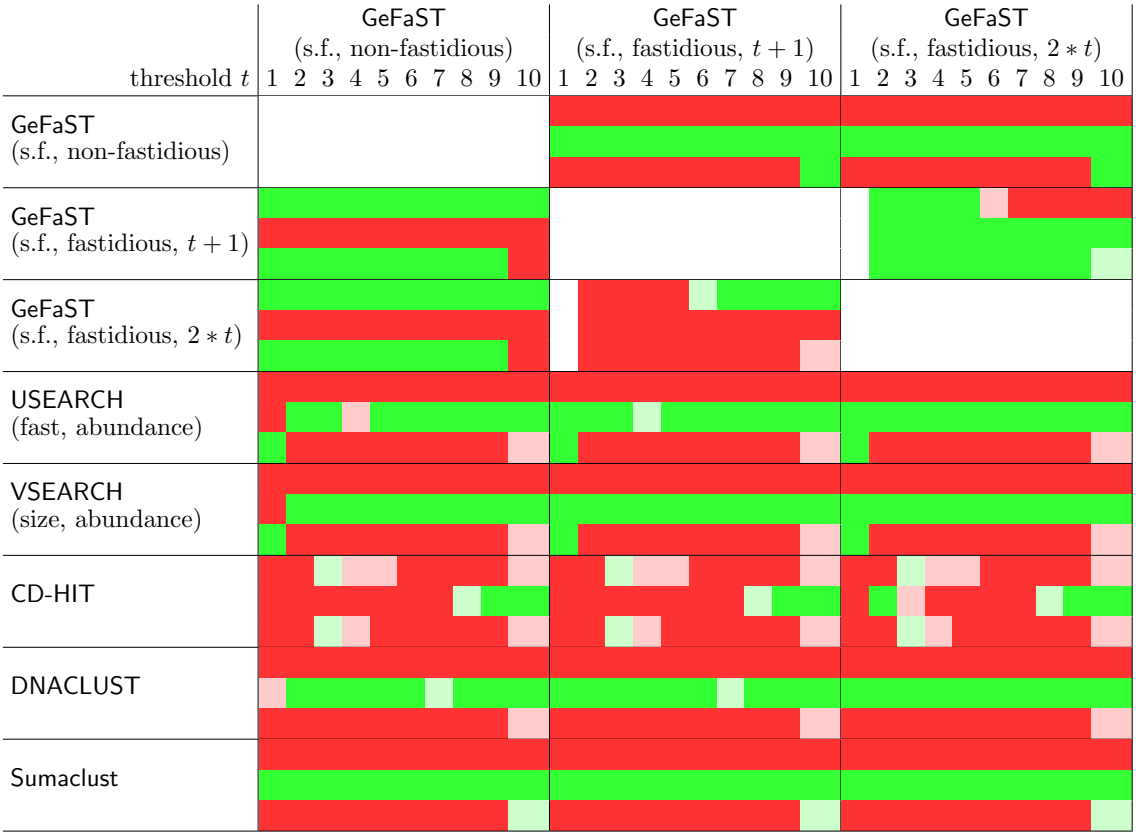
Supplement Table 2 (`uneven`) and Supplement Table 3 (`even`) show the results for the mock-community data, while Supplement Table 4 covers `eldermet`. In these tables, we abbreviated scoring function and edit distance as s.f. and e.d., respectively. The complete information underlying these tables are available in CSV format in Additional file 2 to 4. For each comparison, they state the mean and standard deviation of the differences in the respective metric as well as the p-value. In addition, the size of the mean difference was assessed by comparing it to the standard deviation of the differences (*power1*) and the mean value of the metric (*power2*).



Supplement Table 2: Statistical significance of differences in clustering quality on `uneven`.



Supplement Table 3: Statistical significance of differences in clustering quality on `even`.



Supplement Table 4: Statistical significance of differences in clustering quality on `eldermet`.

References

[1] Mahé, F., Rognes, T., Quince, C., de Vargas, C., Dunthorn, M.: Swarm: robust and fast clustering method for amplicon-based studies. PeerJ **2**, 593 (2014). doi:10.7717/peerj.593

[2] DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L.: Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. Applied and Environmental Microbiology **72**(7), 5069–5072 (2006). doi:10.1128/AEM.03006-05

[3] Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F.: VSEARCH: a versatile open source tool for metagenomics. PeerJ **4**, 2584 (2016). doi:10.7717/peerj.2584

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