Swarm v2: highly-scalable and high-resolution amplicon clustering

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ABSTRACT

Previously we presented Swarm v1, a novel and open source amplicon clustering program that produced fine-scale molecular operational taxonomic units (OTUs), free of arbitrary global clustering thresholds and input-order dependency. Swarm v1 worked with an initial phase that used iterative single-linkage with a local clustering threshold ($d$), followed by a phase that used the internal abundance structures of clusters to break chained OTUs. Here we present Swarm v2 that has two important novel features: 1) a new algorithm for $d=1$ that allows the computation time of the program to scale linearly with increasing amounts of data; and 2) the new fastidious option that reduces under-grouping by grafting low abundant OTUs (e.g., singletons and doubletons) onto larger ones. Swarm v2 also directly integrates the clustering and breaking phases, dereplicates sequencing reads with $d=0$, outputs OTU representatives in fasta format, and plots individual OTUs as two-dimensional networks.

Keywords: environmental diversity, barcoding, molecular operational taxonomic units

INTRODUCTION

Traditional de novo amplicon clustering methods that can handle large high-throughput sequencing datasets (e.g., Edgar, 2010; Ghodsi et al., 2011; Fu et al., 2012) suffer from two fundamental problems. First, they rely on an arbitrary fixed global clustering threshold to group amplicons into molecular operational taxonomic units (OTUs). Global clustering thresholds have rarely been justified and are not applicable to all taxa and marker lengths (e.g., Caron et al., 2009; Dunthorn et al., 2012; Nebel et al., 2011). Second, there is variability in the clustering results due to amplicon input order (Koeppel and Wu, 2013; Mahé et al., 2014).

To solve these problems, we previously introduced the open source Swarm v1 that implemented an initial clustering phase written in C++, then a breaking phase written in Python (Mahé et al., 2014). Swarm’s clustering phase (Fig. 1a) was novel in its approach to single linkage clustering in that, instead of using a global clustering (e.g., Hartmann et al., 2012; Huse et al., 2010), amplicons were iteratively added together using a small local clustering threshold ($d$) until no more amplicons could be added. Using $d=1$ produced the highest resolution OTUs. Swarm’s breaking phase (Fig. 1b) was novel in that it used the abundance of amplicons to reveal the internal structure of potentially chained OTUs (i.e., a low abundant link between high abundant amplicons). These chained OTUs were then refined by splitting them.

Since its introduction, Swarm v1 has been used in a variety of datasets (de Vargas et al., 2015; Filker et al., 2015; Lima-Mendez et al., 2015; Mahé et al., 2015; Oikonomou et al., 2015). However, since the breaking phase was written in Python, it lacked scalability and was cumbersome to use. Kopylova et al. (prep) also found that in comparison to other clustering methods, Swarm v1 tended to produce relatively more low abundant OTUs; e.g., singletons and doubletons. And most importantly, Swarm v1 and other current de novo algorithms could not cluster today’s largest high-throughout sequencing datasets within...
a reasonable amount of time (Rideout et al., 2014). Here we introduce Swarm v2 to help solve these problems, as well as introduce new and useful features.

MATERIAL AND METHODS

Linear complexity de novo clustering approach
Today’s largest amplicon datasets contain hundreds of millions of amplicons and pose a computational challenge to de novo clustering methods. Because of this scalability problem, Rideout et al. (2014) proposed using a mixed clustering approach with an initial closed-reference clustering that compares the amplicons to what is known in taxonomic reference databases to capture most of the data, followed by a de novo clustering with the remaining amplicons. We feel that using only de novo clustering is the most powerful approach when working with amplicons from unexplored environments that lack sufficient taxonomic reference databases or with rare taxa that were previously missed in already-sampled environments. We therefore worked to improve Swarm’s scalability.

Like other current de novo clustering approaches, Swarm v1 presented an apparent quadratic behavior in that it needs to perform a number of comparisons that grows as the square of the number of amplicons. In Swarm v2 we first reduced computational time by improving the multithreading and making a better usage of multi-core CPUs. We further reduced computational time by using a novel algorithmic approach. This linear complexity approach only applies for d = 1, which is Swarm’s default and preferred parameter as it produces the highest resolution clusters.

As background to this linear approach, let us consider a nucleic sequence S made of As, Cs, Gs and Ts. A “microvariant” is a sequence with one difference (d = 1) to the original sequence S. How many distinct microvariants can derive from S? In a sequence S of length L, each position can be substituted with 3 different bases, so there are 3L possible microvariants due to substitutions. Each position in S can be deleted once, so there are L possible microvariants due to deletions. Insertions are more complicated. An insertion can happen before or after each position in the sequence S, and four different nucleotides can be inserted resulting in 4(L + 1) microvariants. However, some insertions will result in the same microvariant: for example, inserting a “G” before or after a “G” will result in the same sequence “GG”. As that situation arises for all positions in S but one, the maximum number of distinct insertions is not 4(L + 1), but 3(L + 1) + 1 = 3L + 4. In total, the maximum number of microvariants that can be obtained from a given sequence S of length L is 3L + L + 3L + 4 = 7L + 4.

As stated above, different sequence modifications can produce the same microvariant. The final number of distinct microvariants depends on the number of homopolymer stretches in the sequence. In the extreme situation where the sequence is entirely made of one type of nucleotide, the number of microvariants due to deletions drops from L to 1. For example, if S is entirely made of “G”, all possible deletions yield the same microvariant. The total number of distinct microvariants then drops to its minimum value: 3L + 1 + 3(L + 1) + 1 = 6L + 5.

The number of distinct microvariants that can be obtained from a sequence S of length L then varies from 6L + 5 to 7L + 4. In practice, it means that a typical high-throughput sequencing 16S rRNA sequence of 130 nucleotides will yield at least 785 microvariants and at most 914, and that the number of microvariants will increase linearly with the sequence length. With current sequencing technologies read length increases at a slower rate than read number, and is safe to assume it will continue to do so in the foreseeable future.

Based on these characteristics of microvariants, we switched from an approximate-string comparison approach to an exact-string comparison approach. That is, for a given amplicon, instead of doing an exact pairwise alignment comparison against all available amplicons in the pool that have yet to be subsumed into an OTU, Swarm v2 generates all possible microvariants of the amplicon and checks whether or not they are present in the amplicon pool using a hash table. This exact-string search approach is extremely fast, and does not depend on the number of available amplicons in the pool. Therefore, the apparent computational complexity changes from n² to n × L, where L is the average amplicon length.

Reducing under-grouping
As observed by Kopylova et al. (prep), Swarm v1 tended to produce relatively more low abundant OTUs; e.g., singletons and doubletons. This problem is due to Swarm’s approach that depends on the existence of a continuous path of linked amplicons. Linking amplicons can be missing, especially when sequencing
is shallow. When this occurs, there can be under-grouping of closely related amplicons leading to small OTUs surrounding a larger OTU.

To address this problem in Swarm v2, we introduced a new step—called the fastidious option—to graft low abundant OTUs onto more abundant ones by postulating a linking amplicon (Fig. 1c). In practice, microvariants (independent of the microvariants produced in the clustering phase) are produced for all the amplicons belonging to low abundant OTUs and stored in a Bloom filter (a probabilistic dictionary). An OTU abundance lower than 3 is the default threshold value (user-controllable parameter); i.e., the fastidious option will target OTUs with an abundance of one (singletons) or two (doubletons). Microvariants are then produced for high-abundant amplicons and cross-checked against the Bloom filter. The fastidious option can consume a large amount of memory, but is apparently linear in terms of computation time (see Results). The user does have control over memory usage and can exchange memory space for computation time. When using $d = 1$, the fastidious option is highly recommended.

The fastidious option can be viewed as a way to reduce data loss, as many researchers conservatively consider low abundant OTUs as spurious errors and remove them from downstream analyses (Behnke et al., 2011; Kunin et al., 2010). With the fastidious option, though, one can retain many of these amplicons by attaching them to more abundant OTUs. In contrast with an increase of $d$, the fastidious option does not degrade the clustering resolution; i.e., it reduces the under-grouping of amplicons without inducing over-grouping.

**Other new and useful features**

In Swarm v2 we introduce a number of options improving both speed and usability. First, there is a simpler user command line interface. For example, the breaking phase is now written in C++ and is performed directly during the growth phase, which further significantly reduces computation time. We chose to implement a strict, simple, non-parametric breaking model that prevents any increase in abundance along a continuous amplicon path (Fig. 1b). Breaking of linked chains can be deactivated.

Second, Swarm v2 extends the notion of clustering by allowing the option $d = 0$. Users can now dereplicate their sequencing reads into strictly identical amplicons (sensu Mahé et al. (2015); i.e., reads that have exactly the same sequences with no mutations, insertions, deletions). This fast dereplication approach uses the same algorithm as in VSEARCH (https://github.com/torognes/vsearch).

Third, Swarm v2 can output OTU representative amplicons in fasta format. A representative is the most abundant amplicon of an OTU, and its abundance is updated to reflect the total OTU abundance. OTU representatives are normally used for downstream community-comparative, novel-diversity, and ecosystem-functioning questions.

Fourth, Swarm v2 offers the possibility to visualize the internal structure of OTUs, which allows the user to gain further knowledge of its underlying genetic and ecological diversity (Fig. 2, 3). These plots are in the form of a network projected in two-dimensions. Edges in these networks only represent the parameter $d$ used; the length of the edges carries no information. The nodes in the networks represent amplicons. The abundance information of these amplicons is represented in three ways: the size of the node, the color of the node, and text when its abundance value is 10 or more.

**Analyses**

To demonstrate the apparent linear complexity of Swarm v2, we analyzed 16S rRNA reads from the Earth Microbiome Project (Gilbert et al., 2014), which is the largest amplicon dataset currently available. The following swarm commands were used: `swarm -d 1 in.fasta`, and `swarm -d 1 -f in.fasta`. To illustrate over- and under-grouping of amplicons, the importance of the breaking phase, high-resolution clustering, and Swarm’s ability to visualize OTUs’ internal structures, we used 18S rRNA amplicon data from the BioMarKs consortium (Logares et al., 2014) that sampled European near-shore marine sites. The PR2 v203 reference database was used for taxonomic assignment (Guillou et al., 2013). The full methods can be found online in html format (Supplementary File 1).

**RESULTS AND DISCUSSION**

**Time and space benchmarks**

For $d = 1$, Swarm’s default parameter, using the full- and sub-datasets of the Earth Microbiome Project we were able to evaluate Swarm v2’s clustering time and memory usage. These timing experiments were obtained with Swarm v2.1.1 on a machine with 1024 GB of RAM running Red Hat CentOS v6.6 and Linux.
We are currently working on a number of fronts to continue making Swarm harder, better, faster, stronger.

We provide examples of Swarm v2’s graphical representation of the internal structure of its high-resolution metadata with the widely used global clustering threshold of 97% similarity, again masking meaningful biological data. When clustering at two OTUs by using V4 and V9 18S rRNA amplicons. In both cases the breaking phase and fastidious option were applied to these V4 amplicons, seven separate OTUs would have been formed: two high abundant, and four low abundant. These three high abundant OTUs are assigned to different genera of Collodaria (Radiolaria). On the same V9 amplicons, UCLUST v6 (as well as v7 and v8) using a global clustering threshold of 97% similarity produced 37 OTUs (one high abundant, and 36 low abundant). The one high abundant OTU from UCLUST lumped the two Collodaria genera, thus masking meaningful biological data.

With the V4 amplicons (about 380 bp in length), the graph shows three high abundant OTUs linked by one to three low abundant amplicons (Fig. 3). The number of nucleotide differences between these three linked OTUs is only two, or about 98.4% similarity. If the breaking phase and fastidious option were applied to these V9 amplicons, nine separate OTUs would have been formed: two high abundant, and seven low abundant. These two high abundant OTUs are taxonomically assigned to different genera of Collodaria. On the same V4 amplicons, UCLUST v6 (as well as v7 and v8) using a global clustering threshold of 97% similarity produced 37 OTUs (one high abundant, and 36 low abundant). The one high abundant OTU from UCLUST lumped the two Collodaria genera, thus masking meaningful biological data.

These amplicon data show that, compared to UCLUST, Swarm v2 can distinguish higher-resolution clusters and reduces both over-grouping and under-grouping on a range of marker lengths. In both of these amplicon examples, Swarm v2 is able to distinguish different taxa, while UCLUST conceals them.

**Outlook**

We are currently working on a number of fronts to continue making Swarm harder, better, faster, stronger. For example, preliminary experiments indicate that with a novel multithreading approach for \( d \geq 2 \) a ten-fold increase in speed could be obtained (although \( d \geq 2 \) will still be quadratic in behavior). Internally
encoding nucleotides on two bits instead of eight bits may help reduce both memory consumption and computational time. Additional computation time can be saved by merging the fastidious option with the initial clustering phase. To facilitate its usage, Swarm v2 can be included in QIIME (Caporaso et al., 2010), which already offers Swarm v1.2, and in Galaxy (Goecks et al., 2010).

In summary, Swarm v2 is a highly-scalable approach that uses a local clustering threshold to produce high-resolution de novo OTUs and reduces low abundant OTUs. Swarm v2 is an optimized C++ program able to handle many hundreds of millions of amplicons. It is open source and freely available at https://github.com/torognes/swarm under the GNU Affero General Public License version 3.

ACKNOWLEDGMENTS

We would like to thank the Earth Microbiome Project for the use of their data and their constructive comments. Daft Punk provided the background music. We are grateful to the computational resources at the Regional Computing Center at the University of Kaiserslautern, and the Abel computing cluster at the University of Oslo.

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**Figure 1.** Schematic view of Swarm’s clustering and refinement approach. (A) Swarm clusters amplicons iteratively by using a small user-chosen local threshold, $d$, allowing OTUs to grow to their natural limits, where no other amplicons can be added. (B) Swarm takes into account the abundance of each amplicon to produce higher resolution clusters, by not allowing the formation of amplicon chains. The darker the red, the higher the abundance. (C) The fastidious option avoids under-grouping (e.g., the production of small OTUs such as singletons and doubletons) by postulating the existence of virtual linking amplicons to graft smaller OTUs onto larger ones.
Figure 2. Graphical representation of an OTU produced by Swarm (breaking and grafting phases deactivated) when clustering the BioMarKs 18S rRNA V9 dataset (amplicons are appr. 129 bp in length). Nodes represent amplicons. Node size, color and text annotations represent the abundance of each amplicon. Edges represent one difference (substitution, deletion or insertion); the length of the edges carries no information. The edge colored in red indicates the breaking point between the two major sub-OTUs, each being assigned to a different genus of Collodaria (Radiolaria).
Figure 3. Graphical representation of an OTU produced by Swarm (breaking and grafting phases deactivated) when clustering the BioMarKs 18S rRNA V4 dataset (amplicons are appr. 380 bp in length). Nodes represent amplicons. Node size, color and text annotations represent the abundance of each amplicon. Edges represent one difference (substitution, deletion or insertion); the length of the edges carries no information. The two edges colored in red indicate the breaking point between the three major sub-OTUs, each being assigned to a different taxa of Cnidaria (Metazoa).