

Starcode: an exact algorithm for sequence clustering

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ABSTRACT

Motivation: The increasing throughput of sequencing technologies offers new applications and challenges for computational biology. One such application is the use of random barcodes to trace and quantify transcripts or lineages in experimental setups. The high error rate of modern sequencers calls for additional post-processing techniques capable of detecting and reverting the misreads. However, in the absence of a reference population, the problem amounts to performing a pairwise comparison of all the barcodes, which is unfeasible for excessive computational complexity.

Results: Here we address this problem and describe an exact algorithm to determine which pairs of sequences lie within a given Levenshtein distance. The matched pairs are merged into clusters represented by a canonical sequence. The efficiency of starcode is attributable to the poucet search, a novel implementation of the Needleman-Wunsch algorithm performed on the nodes of a trie. Parallelization achieves linear scaling of performance on multi-core machines. On the task of clustering random barcodes, starcode outperforms short read mappers and sequence clustering algorithms in both speed and precision.

Availability and implementation: The C source code is available at <http://github.com/gui11aume/starcode>.

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1 INTRODUCTION

Sequence clustering is the process of grouping similar biological sequences. It has been traditionally applied to identify related protein families and to reduce sequence redundancy in databases. Recently, the advent of high throughput sequencing has created additional needs for efficient clustering algorithms, in particular because of the high error rate of such technologies. For instance, the Illumina platform (Margulies *et al.*, 2005) shows a 1-2% error rate consisting of substitutions near the 3' end of the read (Dohm *et al.*, 2008; Nakamura *et al.*, 2011). The PacBio platform shows a 15% error rate that mostly corresponds to insertions and deletions (Eid *et al.*, 2009). As a consequence, the same sequence is often decoded in different ways, which artificially increases the diversity of the output.

Sequencing errors can be discovered by mapping the reads onto a reference, if it is available. When the sequences are random or drawn from an unknown reference, clustering is the best option to

tell real from spurious reads. One such case is the use of random barcodes to track cells or transcripts (Schepers *et al.*, 2008; Akhtar *et al.*, 2013). Sequencing errors will create erroneous barcodes that have to be reverted to the original sequence. Hands on experience with real datasets show that this step becomes limiting when the number of unique sequences is high. In search for a solution to this problem, we realized that heuristic approaches rely on assumptions that may not hold as technologies evolve. We therefore set out to find an exact algorithm.

The first task of clustering is a matching phase where closely related barcodes are paired, similarly to linked nodes on a graph. The second task is the clustering proper, where communities in this graph are merged. We called our algorithm “starcode”, in reference to the star shape of the graph formed by the barcodes in the same cluster. The first version of the algorithm had the same performance regardless of the order in which the input sequences were processed, because it did not exploit data structuring of any kind. Exploiting the prefix redundancy of alphabetically sorted sequences allowed us to avoid unnecessary recomputations and gain speed. This is the rationale behind the poucet search algorithm at the heart of the matching step.

Here we describe the starcode algorithm and we benchmark it against existing software that perform related tasks. We show that starcode is both faster and more precise than the alternatives, achieving perfect clustering on ideal datasets. We further show that starcode is a good candidate to cluster k-mers from large datasets from bacterial or even metazoan genomes.

2 METHODS

2.1 Inexact string matching using tries

The matching method of starcode is based on a variation of the Needleman-Wunsch (NW) algorithm (Needleman and Wunsch, 1970). In the original algorithm (Figure 1a), the Levenshtein distance between two sequences is found by applying a recurrence relation throughout a matrix of mn terms, where m and n are the respective sequence lengths. The complexity of this dynamic programming approach is $O(mn)$.

In many instances, the only information of interest is to find out whether the sequences are d -neighbors (separated by a distance less than or equal to a threshold d). In that case, the complexity then reduces to $O(d \min(m, n))$ as described below (Ukkonen, 1995). Instead of initializing the margins of the matrix and computing all the terms, the matrix is initialized as shown on Figure 1b and only the terms around the diagonal are computed. If the computation of a diagonal term yields a value greater than d , the distance is

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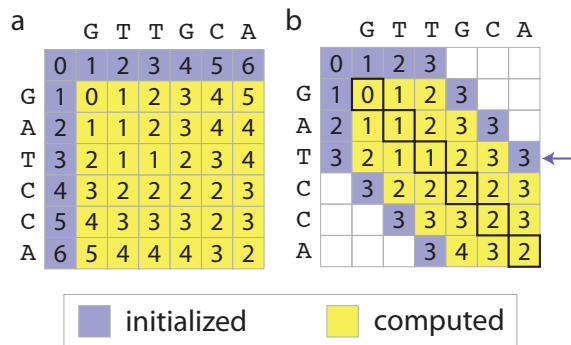


Fig. 1. Needleman-Wunsch (NW) alignment. **a** Alignment of GTTGCA and GATCCA using the NW alignment matrix. The margins (purple) are initialized and the cells of the matrix (yellow) are computed from left to right and from top to bottom by the following dynamic programming algorithm. The value in each cell is computed from the values of the neighboring top, left and top-left cells. Denoting a , b and c these respective terms, the value in the cell of coordinates (i, j) is computed as $\min(a + 1, b + 1, c)$ if the i -th letter from the first sequence is identical to the j -th letter from the second, or $\min(a + 1, b + 1, c + 1)$ if the letters are different. The Levenshtein distance between the two sequences is found in the bottom right cell. **b** Lower complexity algorithm to determine whether GTTGCA and GATCCA are 2-neighbors. The values in purple cells are set during initialization. The dynamic programming algorithm proceeds as above, with the difference that it is interrupted if the value of a diagonal cell (bold borders) is larger than 2. The values in purple cells are not always the same as their equivalent in a (purple arrow), but the values in the yellow cells are nevertheless identical. The values of the white cells are never computed, which contributes to making the algorithm less complex.

known to be greater than d and the process is halted. Otherwise, the bottom-right term indicates the actual distance between the sequences, as in the NW algorithm.

This method can be used for inexact matching of sequences against a reference set indexed as prefix tree, also known as a trie (Ukkonen, 1995). The terms of the matrix are updated row-wise as a depth-first search traverses the trie from the root, as illustrated in Figure 2. Every time a node is visited, the row corresponding to its depth is recomputed. If the threshold value d is exceeded for a diagonal term, the Levenshtein distance for all the downstream sequences is also necessarily greater than d . Therefore, no more hits are to be discovered in this path and the depth-first search backtracks to the parent node. When the process halts, every tail node (corresponding to a sequence of the database) on the path of this search is a d -neighbor of the query. This method is efficient because it eliminates large areas of the search space, and because the NW alignment of the query with each prefix of the database is computed only once.

2.2 The poucet search algorithm

This strategy can be further improved. Notice that if two consecutive queries share a prefix of length k , the succession of computations up to the k -th row of the NW matrix will be exactly the same in both queries. To take advantage of this property, the input sequences are sorted alphabetically in order to maximize the prefix sharing. The main idea of the algorithm is to store the computational intermediates in the nodes of the trie and use them later to resume the computation in the next query. This way, the search can start at depth k whenever a query shares a prefix of such length with the previous one.

However, storing the rows of the NW matrix in the nodes, as suggested by the approach described in the previous section is not optimal. At depth

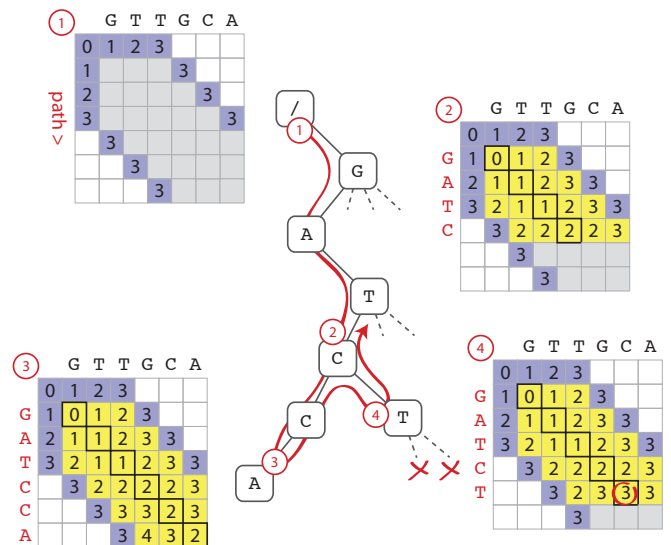


Fig. 2. Trie query with NW alignment. Each sequence of the index is a path in the trie. The query GTTGCA is written at the top of a NW matrix, which is initialized as shown on Figure 1b. The trie is traversed by a depth-first search (red path) from the root. At each depth, the node added to the path is written on the left of the NW matrix and the row is computed. Checkpoints from 1 to 4 (circled red numbers) show the state of the NW matrix as the search proceeds. The node labelled 3 is a leaf and thus corresponds to a 2-neighbor of the query. The search path then backtracks to the node labelled 2 and the last rows of the NW are erased. The search path then goes to node labelled 4, in which case the newly computed diagonal cell exceeds the threshold (circled in red). Even if this node has children, they are not visited (red crosses).

equal to the length of the shared prefix, say k , the terms on the right of the diagonal were computed using characters that belong to the previous query. Since they also depend on the terms computed at lower depth, the search cannot restart at depth k . This issue is solved by storing in each node a combination of row and column terms that form an angle shape, looking like a horizontally flipped L character as shown on Figure 3. This way, the computation intermediates stored in a node of depth k depend only on the previous characters of the query. Using this technique, the search can effectively restart at a depth equal to the length of the shared prefix with the previous query, thereby averting the need to recompute the first terms of the NW alignments.

In the fairy tale “Le Petit Poucet”, the hero seeds white pebbles for his older brothers to find their way home, which is reminiscent of the way queries pave the way for the next in this algorithm. We therefore called this search algorithm “poucet”.

2.3 Lookup search

If the Levenshtein distance between two sequences of length L is less than or equal to τ , then there must exist a perfect match of length at least $k = L/(\tau + 1)$, assuming for the time being that k is an integer. We take advantage of this property to build a lookup table containing all such k -mers from sequences inserted in the trie. When a new sequence is queried, subsequences are first extracted from it and queried against the lookup table. If none of the subsequences are found, the minimum distance between the query and all the sequences in the trie is at least $\tau + 1$, and the trie-search can therefore be omitted. This lookup is particularly useful for long query sequences because in this case k is large. The probability of an exact match

Algorithm 2 Poucet search algorithm

```

1: procedure POUCKET(query, node, seed, hits):
2:   COMPUTE node-specific column values following NW
   algorithm ▷ Fig.1
3:   for all child nodes in node do
4:     COMPUTE child-specific row values following NW
   algorithm ▷ Fig.1
5:     COMPUTE center value using row and column values ▷ Fig.1
6:     if center value >  $\tau$  then ▷ Mismatches exceeded.
7:       continue with next child
8:     end if
9:     if node depth = height then ▷ Hit found.
10:      SAVE node sequence in hits
11:      continue with next child
12:     end if
13:     if node depth ≤ seed then
14:       SAVE node in pebbles at current depth
15:     end if
16:     call poucet(child, seed, hits)
17:   end for
18: end procedure

```

corresponding canonical barcode. The barcodes for which the read counts are split between different canonical barcodes are not assigned to any cluster. The reason for imposing a factor 5 or larger in order to transfer the read counts is that barcodes with similar frequencies are not likely derived from each other through sequencing errors. More likely they are either unrelated, or they both derive from another more abundant barcode.

2.7 Benchmark options

The benchmark dataset is described in the Results section. All software were set to run with 20 parallel threads on a 20-core Intel Xeon processor. The execution options for each software are summarized in Table 1.

Table 1. Software execution parameters

starcode	-t 20
CD-HIT 4.6.1	-c 0.925 -T 20 -M 0
USEARCH 7.0.1090	-threads 20 -cluster.fast -id 0.925
Bowtie2 indexer	bowtie2-build --quiet
Bowtie2 2.1.0	-f -a -p 20 --no-hd --very-sensitive
BWA indexer	bwa index -a is
BWA 0.7.9a	mem -t 20 -a -k1 -B0
GEM indexer	gem-indexer -T 20
GEM 1.423	-e3 -s -T 20 --granularity 100000
<i>sequential R</i>	method='lv', maxDist=2

Denoted as *sequential R* is the sequential algorithm of Akhtar *et al.* (2013) which is implemented in R and relied on the `stringdist` function of the `stringdist` package.

3 RESULTS

3.1 Benchmark against sequence clustering algorithms

Sequence clustering is routinely used to curate databases of non redundant nucleotide or protein sequences. We benchmarked starcode against the two popular sequence clustering algorithms CD-HIT (Fu *et al.*, 2012) and USEARCH (Edgar, 2010).

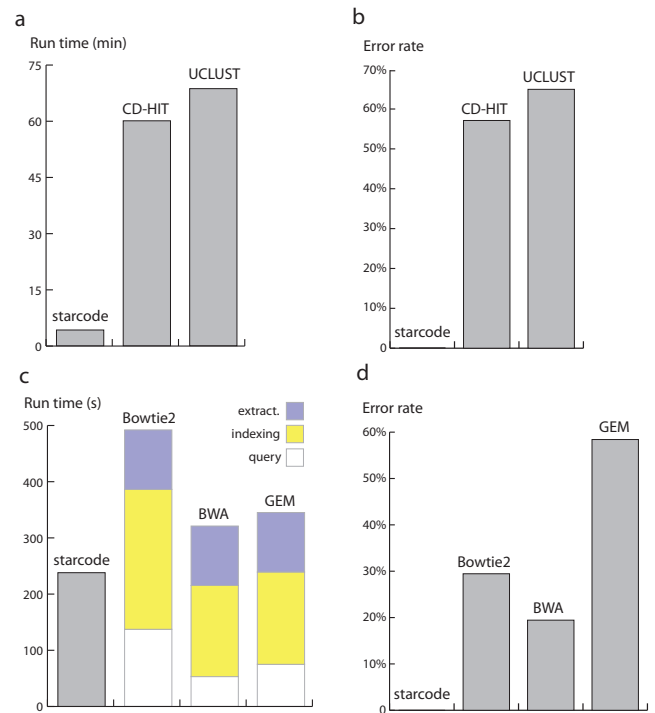


Fig. 4. Benchmark of starcode against sequence clustering algorithms and short read mappers. **a** Running time compared to CD-HIT and UCLUST on the dataset described in the text. **b** Error rate compared to CD-HIT and UCLUST, expressed as the percentage of clusters failing to be merged. **c** Running time compared to Bowtie2, BWA and GEM on this dataset. The running time is decomposed in extraction of unique sequences (purple), Burrows-Wheeler indexing (yellow), and query proper (white). The extraction was performed with the Linux sort command, so the time is the same in every case. **d** Error rate compared to Bowtie2, BWA and GEM, expressed as the proportion of matches with a single hit. This is an underestimate of the true error rate.

We generated 1 million random 40-mers that were duplicated 47 times. For each 40-mer, we inserted 3 additional mutant sequences to the pool by sampling again 3 nucleotides at random positions. The probability that such a mutant sequence is within a distance 3 of any other 40-mer is of the order of 10^{-12} and can be neglected. Each cluster thus consists of 50 sequences, of which 3 have a distance at most 3 from the canonical representant.

Using 20 cores, starcode clustered the shuffled 50 million sequences in 4 minutes without a single error (the output consisted of 1 million clusters of size 50). We submitted the unique sequences of the dataset to CD-HIT and USEARCH. The results are summarized in Figure 4a and 4b. The running times of CD-HIT and USEARCH were 59 and 64 minutes respectively, and they identified 2,362,352 and 2,739,954 clusters respectively. Both algorithms rely on heuristics, fact that explains why the number of clusters is off target. These results clearly demonstrate that starcode outperforms other clustering algorithms in speed and accuracy on this particular task.

3.2 Benchmark against short read mappers

A potential strategy to cluster short sequences is to use last generation short read mappers to match a set of sequences against itself. Once matches are available, several community detection algorithms can be used to identify the clusters. We benchmarked starcode against the short read mappers Bowtie2 (Langmead and Salzberg, 2012), BWA (Li and Durbin, 2009) and GEM (Marco-Sola *et al.*, 2012). Because short read mappers do not perform clustering, we only evaluated their performance on the matching problem.

For this test, we used the same dataset described above, in which every sequence has at least 2 distinct matches at a distance 3 (mutant sequences from the same cluster may be at a distance up to 6). This means that we can use matches with a single hit (themselves) as a lower bound on the inaccuracy. We used unique sequences to build an index and queried the index with the same sequences. Figure 4c shows the running time of starcode along with the decomposition of the running time for the three mappers. Note that the time to extract unique sequences is always the same because we used the `Linux sort -u` command in every case. It appears that the running time of starcode is about the same as the time required by BWA (the fastest aligner in this test) to index and run the query proper. Figure 4d shows the lower bound on the error rate, as estimated by the proportion of single hits. BWA was found to be the most accurate aligner with a lower bound of 19% errors. This measure is indeed an underestimate of the true error rate, but given that starcode makes no mistake on this dataset, a more accurate measure is not necessary. In conclusion, starcode runs slightly faster than short read mappers when all steps are taken into account, and clearly outperforms them in precision on this particular problem.

3.3 Clustering TRIP barcodes

In the course of setting up the TRIP technology in our laboratory (Akhtar *et al.*, 2013), we realized the need to develop efficient algorithms to cluster similar sequences. Briefly, the principle of TRIP (Thousands of Reporters Integrated in Parallel) is to tag reporter transcripts with random barcodes and measure the abundance of barcodes in the RNA as a proxy for gene expression. There is no reference to match aberrant barcodes against, because the tagging sequences are unknown. Instead, barcodes are matched against each other and clustered by similarity to infer canonical sequences.

We tested the efficiency of starcode on the TRIP dataset from Akhtar *et al.* (2013). In the experiment labelled mPGKA, we identified 24.1 million (91%) barcode-containing reads out of 26.6 million, consisting of approximately 223,000 and 220,000 unique barcode sequences for PCR replicates 1 and 2, respectively. Following the authors, we kept only the barcodes with at least 5 counts and performed clustering as described: “First we sorted barcodes according to their counts. Then, for each barcode (starting from the most frequent one), we identified and removed all its mutant versions, defined as barcodes within a Hamming distance of 2.” This method is here on referred to as the “sequential algorithm”. When replacing the Hamming distance by the Levenshtein distance, starcode produced exactly the same output as the sequential algorithm. The running time of starcode averaged over the replicates was 2.90 seconds, *versus* more than 3 minutes 40 seconds, which represents a 75-fold speedup.

The performance of the sequential algorithm relies on the arbitrary exclusion of reads with less than 5 counts, the main purpose of which is to reduce the computational burden. When all barcodes were kept, the average running time of starcode was 10.27 seconds, *versus* > 6 hours for the sequential algorithm. Using the Hamming distance, as the authors originally suggested, decreased the average running time of the sequential algorithm to about 3 hours and 30 minutes (and the output differed from that of starcode). Note that in all the cases mentioned above, starcode was run with a single core to compare the algorithms based on similar computer resources. In conclusion, the barcode clustering problem can be simplified by various tricks, but starcode brings down the running time to nearly instantaneous, and thereby obviates the need for such arbitrary heuristics.

3.4 Identifying enriched sequence motifs

Sequence motifs are thought to play an important role in DNA metabolism. Key regulators, such as transcription factors, nucleosomes and non coding RNAs have sequence preferences targeting them to the sites where they act. Identifying those sequences is a way to pinpoint the regulators and the mechanisms they are involved in. However, the sequence motifs are not strictly identical at different sites, hence they are better identified by inexact matching. This problem becomes computationally difficult for long motifs (above 12-13 nucleotides) because of the combinatorial scaling. But as motifs become longer, the problem of identifying abundant inexact matches becomes similar to barcode clustering.

We set up a test based on the meningitis-causing agent *Neisseria meningitidis*. The genome of this bacterium is interspersed with a frequent 12 bp sequence known as DNA uptake sequence (Smith *et al.*, 1999). We extracted the 12-mers from both orientations of the 2.19 Mb genome, yielding 4.39 million 12-mers, consisting of 2.77 million unique sequences. Clustering the 12-mers with starcode within a Levenshtein distance of 2 took less than 3 minutes with 20 cores. We identified the known DNA uptake sequence of *Neisseria meningitidis* (ATGCCGTCTGAA) as the most abundant 12-mer, comprising 1,466 exact matches and 4,625 inexact matches. This result testifies to the fact that starcode can be used to identify biologically relevant motifs in bacterial genomes.

To test starcode on the scale of a metazoan genome, we used a *Drosophila* dataset from Filion *et al.* (2010). The study describes two signatures of chromatin proteins called Red and Yellow, both corresponding to transcribed regions of the genome. The distinction is based on epigenomic features only, which prompted us to identify sequence features that could discriminate Red *versus* Yellow regions. To this end, we used starcode to cluster 18-mers from both regions.

The total coverage of Red and Yellow chromatin in the *Drosophila* genome is 10.7 Mb and 21.3 Mb respectively, corresponding to 21.5 million and 42.5 million 18-mers, respectively. We used starcode to identify clusters of 18-mers within a Levenshtein distance of 3 (the running time was approximately 35 minutes for Red and 70 minutes for Yellow chromatin using 20 threads). The most discriminating 18-mer was (GA)₉, which showed a 4.8 fold enrichment in Red *versus* Yellow chromatin. We counted 832 exact matches and 6,749 inexact matches over 3,880 distinct 18-mers in Red chromatin, showing that a larger portion of the signal comes from inexact matches. Taken together, these

results show that starcode can be used to cluster short k-mers from metazoan-scale datasets.

4 DISCUSSION AND CONCLUSION

Through the parallel poucet search algorithm, starcode implements an exact sequence clustering algorithm that is even faster than widely used heuristics. By design, starcode is tailored to process high throughput sequencing data on multi-core platforms. Our benchmark shows that starcode is superior in speed and accuracy compared to other methods on the problem of clustering short random sequences. Part of the reason is that such task is stretching the tested software far from their initial design. Sequence clustering algorithms are meant to cluster long biological sequences, while short read mappers are meant to map reads on potentially large genomes. In this respect, starcode fills a need arising from the development of barcoding technologies.

The speed of starcode also makes it proficient at more general clustering problems, such as identifying enriched k-mers in genomes and in experimental data. Here we have given two examples of such applications. In the first, we recover a known enriched 12-mer in the genome of *Neisseria meningitidis*. In the second, we identify a sequence feature that discriminates genomic regions found in two different chromatin types in *Drosophila*. GA repeats are known to be bound *in vivo* by the protein GAF (van Steensel *et al.*, 2003), which suggests that this transcription factor is instrumental in setting the distinction between Red and Yellow chromatin.

The current version of starcode has been mildly optimized for memory consumption. The memory footprint depends on the number of sequences to cluster (because the sequences of the input set are loaded in memory as a group of tries) and on the mean number of matches per sequence. Every match has to be stored until the clustering phase, which may represent a large memory overhead. As counterintuitive as it may seem, long queries will usually impose a lower memory footprint because the matches between sequences are less frequent.

The speed of starcode could also benefit other applications, such as searching shared subsequences during genome assembly. However, starcode supports only global and not local alignment. A workaround is to compare k-mers as we have done in the examples above, but this approach is expected to be memory demanding. Even though extending the starcode algorithm to support clustering of protein sequences is straightforward, it is not expected to meet the performance level of the presented algorithm. On the one hand, there are more letters in the protein alphabet, so the shared prefix between consecutive queries is inherently shorter on average, which defeats the poucet search. On the other hand, if a threshold distance of the order of 1 per 10 nucleotides is sufficient to identify similar DNA sequences, a distance of 1 per 10 residues is unrealistically low for protein sequences, and this parameter critically determines the speed of starcode. More generally, storing computational intermediates for shared prefixes could find some applications in other algorithms such as short read mappers. The idea of the poucet search seems simple in retrospect, but it is a powerful way to tap into the data structuration provided by string sorting.

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