

Sequence analysis

Designing efficient randstrobes for sequence similarity analyses

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Associate Editor: Alfonso Valencia

Abstract

Motivation: Substrings of length k , commonly referred to as k -mers, play a vital role in sequence analysis. However, k -mers are limited to exact matches between sequences leading to alternative constructs. We recently introduced a class of new constructs, *strobemers*, that can match across substitutions and smaller insertions and deletions. *Randstrobes*, the most sensitive strobemer proposed in Sahlin (Effective sequence similarity detection with strobemers. Genome Res 2021a;31:2080–94 <https://doi.org/10.1101/gr.275648.121>), has been used in several bioinformatics applications such as read classification, short-read mapping, and read overlap detection. Recently, we showed that the more pseudo-random the behavior of the construction (measured in entropy), the more efficient the seeds for sequence similarity analysis. The level of pseudo-randomness depends on the construction operators, but no study has investigated the efficiency.

Results: In this study, we introduce novel construction methods, including a Binary Search Tree-based approach that improves time complexity over previous methods. To our knowledge, we are also the first to address biases in construction and design three metrics for measuring bias. Our evaluation shows that our methods have favorable speed and sampling uniformity compared to existing approaches. Lastly, guided by our results, we change the seed construction in strobalign, a short-read mapper, and find that the results change substantially. We suggest combining the two results to improve strobalign accuracy for the shortest reads in our evaluated datasets. Our evaluation highlights sampling biases that can occur and provides guidance on which operators to use when implementing randstrobes.

Availability and implementation: All methods and evaluation benchmarks are available in a public Github repository at <https://github.com/Moein-Karami/RandStrobes>. The scripts for running the strobalign analysis are found at <https://github.com/NBISweden/strobalign-evaluation>.

1 Introduction

In sequence analyses, k -mers play an important role in various algorithms and approaches. For example, k -mers can be used as *seeds* for sequence similarity search, where a seed shared between two sequences acts as an *anchor* to identify similar regions between, e.g. DNA, RNA, or protein sequences. When used as seeds, k -mers enable rapid identification of shared regions and are used in a large number of short and long-read mapping algorithms (Alser *et al.* 2021, Sahlin *et al.* 2023), and other approaches for querying large sequence datasets (Marchet *et al.* 2021, Fan *et al.* 2024).

Both a feature and a limitation of using k -mers as seeds is that sequences must be identical for the seed to match. In biological data, it is common that mutations in DNA occur in the form of substituted, deleted, and inserted nucleotides. In addition, common DNA and RNA sequencing techniques are noisy and introduce additional altering of the nucleic acids. In order to provide anchors also in regions with high divergence, seeds are allowed to *anchor* over mutations. Alternatives to k -mers have therefore been explored extensively in the literature, such as spaced k -mers (Ma *et al.* 2002). See Sahlin *et al.* (2023) for

an overview of several other seeding constructs used in read mapping.

1.1 Strobemers

Recently, we introduced *strobemers*, a novel class of seed constructs (Sahlin 2021a). Strobemers can produce seed matches across substitutions, insertions, and deletions, expanding on ideas from neighboring minimizer pairs (Chin and Khalak 2019, Sahlin and Medvedev 2021) and k -minmers (Ekim et al. 2021) that link neighboring minimizers (Roberts et al. 2004) into a seed. Strobemers generalize this linking by considering downstream k -mers as potential candidates to link, offering various methods such as minstrobemes, randstrobemes, and hybridstrobemes (Sahlin 2021a), with *randstrobemes* being the most effective. Randstrobemes have been used, e.g. in for short-read mapping (Sahlin 2022), transcriptomic long-read normalization (Nip et al. 2023), and read classification (Xu et al. 2023). Our recent study also demonstrates that randstrobemes provide accurate sequence similarity ranking using the Jaccard distance (Maier and Sahlin 2023). This study also revealed a strong correlation between strobemers' sensitivity and the pseudo-randomness of the seed construct, measured through entropy (Maier and Sahlin 2023). While additional strobemer variants have been introduced (Maier and Sahlin 2023), randstrobemes remain the simplest and most widely used construct. Constructing randstrobemes involves converting strings to integers using a hash function and selecting candidate k -mers for linking through a link function and comparator operator. Sampling biases (Fig. 1) in this process can affect sequence matching efficiency (Maier and Sahlin 2023). So far, the underlying operators to produce randstrobemes have not been evaluated.

1.2 Our contribution

We design metrics suitable for detecting and measuring several types of bias in randstrobe construction methods (Fig. 1). Using the new evaluation metrics, we uncovered biases and limitations in previous randstrobe methods (Sahlin 2021a, 2022, Xu et al. 2023). We propose new methods to enhance the core operations (hashing, linking, and comparison), which improve seed uniqueness, sampling uniformity, and construction runtime. We also introduce a Binary Search Tree (BST)-based construction method, reducing time complexity and achieving comparable randomness but is much

faster for some parametrizations. This is valuable for time-critical bioinformatics applications.

Additionally, we identify that the link function and comparator in the short-read mapper strobealign (Sahlin 2022) underperform in seed uniqueness compared to other methods. As a result, we modified strobealign to enhance accuracy. Although the modification does not improve the overall accuracy, an approach that selects the best alignment score per read from the modified and default versions of strobealign improves accuracy substantially. This finding can be used to further increase strobealign's accuracy. In summary, our evaluation uncovers linking biases and offers guidance on operator selection for randstrobe implementations.

2 Materials and methods

2.1 Definitions

We use 0-indexed notation. We typically use S and T to denote strings, and we use the notation $S[i:j]$ $i \leq j$ to refer to a substring starting at position i and ending (and including) the character at position j in S . We let the $|S|$ operator denote the length of strings. Here, our alphabet consists of the letters (or *nucleotides*) $\Sigma = \{A, G, C, T\}$. We use $h(x)$ to denote a hash function without specifying the underlying function. As for representation in memory, DNA strings shorter or equal to 32 nucleotides (nt) can be stored with 64-bit integers by encoding A, C, G, and T as 00, 01, 10, and 11, respectively. Other letters, such as N for "unknown" nucleotide, are ignored. For k -mers longer than 32 nt, we represent them as structs of (concatenated) 64-bit integers. We use the variable x to represent the integer value of the encoding. Finally, we use $\&$ for bitwise AND, $\%$ for bitwise XOR, $\|$ for concatenation (e.g. concatenating two 64-bit integers into a 128-bit representation), and $\%m$ for the modulo operator. We also use $B(x)$ to represent the function that returns the number of set bits in x .

2.2 An overview of constructing strobemers

A k -mer is a substring of k nucleotides in a biological sequence S . Consequently, a k -mer only needs the length of the substring, k , as a parameter to be specified. A strobemer is a set of linked k -mers. Specifically, a strobemer consists of n k -mers $l_0; \dots; l_{n-1}$ denoted *strokes*, where the first stroke l_0 has a determined position i in S . Downstream stroke l_m , $m \geq 1$ is selected in an interval $S[i_0 + w_{min}, i_0 + w_{max}]$:

Figure 1. Illustration of a desired random sampling of the second stroke for strobemers consisting of two strokes (case A). Whenever a pseudo-random method is used to select the downstream stroke based on the first stroke, it generates some sampling bias. Cases B–E show different biases we observed in the sampling. The metrics we propose to measure the bias are displayed under each of the illustrations of cases B–E.

in S , and *linked* (appending the strobe to previous strobes) to the m previous strobes. Here, w_{min} and w_{max} specify the range of the sampling window. For example, strobe l_1 is sampled in $S[l_0 : l_0 + w_{min} - 1]$ and linked to l_0 .

Since we consider 64-bit integer representations of the strobes in this study, we will from now on refer to the strobes as x_0, x_1, \dots, x_{n-1} and, when clear from context, we alternate x to mean either the strobe itself or its integer representation. This is also the reason we use the more general term *linking* instead of *appending* (strobes to the seed), as the linking method will vary with the strobe representation, as we discuss in detail in the next section.

The methods to select strobes differ (Sahlin 2021a). For example, Minstrobes have been used for long-read overlap detection (Firtina et al. 2023) and alternating strobe lengths have also been explored (Maier and Sahlin 2023). However, randstrobes were shown to be more sensitive for sequence matching than other methods using fixed strobe lengths (minstrobes and hybridstrobes) (Sahlin 2021a), and simpler to construct than alternating strobe lengths (altstrobes and multistrobes) (Maier and Sahlin 2023), and is so far most commonly implemented in practice (Sahlin 2022, Nip et al. 2023, Xu et al. 2023). Therefore, we will consider only the randstrobes method in this study. Randstrobes are parameterized by (n, w_{min}, w_{max}) . The novelty compared to, e.g. k -mers and spaced k -mers is that strobemers allow flexibility in the strobes' spacing and can produce matches between two sequences in a region with insertions or deletions.

2.3 Strobemer construction: constraints and objectives

Let $M_{w_{min}, w_{max}}^{x_{i-1}, \dots, x_0}$ or simply M when context is clear, be a method to sample a strobe x_i in a window given by its parametrization (n, w_{min}, w_{max}) . We put the following constraints on M .

- 1) M selects x_i based only on the sequence information of x_{i-1}, \dots, x_0 .
- 2) M is deterministic. That is, for two identical strings S and T , the same strobes are produced.

We want to find a method M such that

- 1) Maximize $H(M_{w_{min}, w_{max}}^{x_{i-1}, \dots, x_0})$ where H denotes the entropy. Intuitively, M should sample x_i as uniform as possible within the window, regardless of previous strobes and the sequence in the window.
- 2) M constructs randstrobes as fast as possible.

The first constraint is essential to eliminate high-entropy but impractical solutions in sequence matching. For instance, using a (pseudo) random number generator (RNG) like `rand()` in C++ may seem to have good entropy. However, in scenarios involving similar strings S and T , where one has a deletion, the RNG is likely to generate different numbers upon encountering the deletion, making it unsuitable for string matching. Therefore, the method's decision should solely rely on the underlying sequence.

The first objective, instead, involves conditional entropy, which is challenging to measure. Merely assessing entropy by the uniformity of sampling sites within a sequence window is insufficient. For instance, if a method prefers selecting a strobe if it is identical to the previous strobe, and the distance

between two identical strobes happens to be uniformly distributed across a sequence, the method may falsely appear to have perfect entropy. It is also worth noting that achieving high entropy is easier in randomly generated sequences, but the focus here is on repetitive regions common in biological sequences, where achieving sampling uniformity is more challenging.

2.4 Constructing randstrobes

The process of creating randstrobes can be separated into four modular components:

- 1) Hashing the strobes;
- 2) Linking the strobes;
- 3) Comparing the strobes during linking;
- 4) Construction of the final seed hash value.

We discuss each of the components below and suggest different methods to perform them.

2.4.1 Hashing strobes

Since each strobe is represented as a 64-bit integer using the binary encoding, the integers can further be hashed. The reason for hashing a strobe x as $h(x)$ is that it can improve the pseudo-randomness. We evaluate the following hash functions for the strobes:

$h_{NO}(x)$ The original 2-bit encoding of nucleotides is used without applying a hash function.

$h_{TW}(x)$ Thomas Wang hash (<http://web.archive.org/web/20071223173210/http://www.concentric.net/~Ttwang/tech/inthash.htm>), an invertible hash function used, e.g. in `minimap2` (Li 2018).

$h_{XX}(x)$ xxHash (<https://xxhash.com/>).

$h_{WY}(x)$ wyhash (<https://github.com/wangyi-fudan/wyhash>).

Previously, $h_{NO}(x)$ was used in Sahlin (2021a) and $h_{TW}(x)$ was used (Sahlin 2022). This is the first study using $h_{XX}(x)$ and $h_{WY}(x)$ hash functions to construct randstrobes. The hash functions xxHash and wyhash are general-purpose non-cryptographic pseudo-random hash functions that hash bytes into an integer range of size 2^b for some $b > 0$ (here, $b \leq 64$).

2.4.2 Linking strobes

The second strobe x_1 is *linked* to the first strobe x_0 by selecting the candidate strobe x_1^d in the window that minimizes or maximizes the link function ℓ . For example, in the first strobemers study (Sahlin 2021a), two link functions were used. The first was $\ell(x_0, x_1^d) = (x_0 \oplus x_1^d) \bmod p$, $p \in \mathbb{Z}$ [originally proposed in the preprint (Sahlin 2021b)]. The second one was $\ell(x_0, x_1^d) = (x_0 \& x_1^d) \& q$, where q is a bitmask of 16 ones' on the lowest significant bits and remaining 0s [proposed as faster alternative in the final publication (Sahlin 2021a)]. We call these functions ℓ_{MOD} and ℓ_{AND} , respectively. Furthermore, two additional link functions were described in Sahlin (2022) and Xu et al. (2023) that we denote ℓ_{BC} and ℓ_{XOR} . Here we propose three more alternatives: ℓ_{XV} , ℓ_{CC} , and ℓ_{MAMD} . We provide formal definitions of all the link functions below.

$\ell_{MOD}(x_0, x_1^d) = (x_0 \oplus x_1^d) \bmod p$, $p \in \mathbb{Z}$ (see Sahlin 2021a)

$\backslash \text{AND} \backslash x: x_1 \backslash h \backslash x \backslash \& q \geq N$ (see Sahlin 2021a)
 $\backslash \text{BC} \backslash x: x_1 \backslash h \backslash x \backslash \% h \backslash x \backslash$ (see Sahlin 2022)
 $\backslash \text{XOR} \backslash x: x_1 \backslash h \backslash x \backslash \% h \backslash x \backslash$ (see Xu et al. 2023)
 $\backslash \text{XV} \backslash x: x_1 \backslash h \backslash x \backslash \% x_1 \backslash$ (proposed in this study)
 $\backslash \text{CC} \backslash x: x_1 \backslash h \backslash x \backslash j x_1 \backslash$ [described in the pseudo code in Sahlin (2021a) but never studied]
 $\backslash \text{MAMD} \backslash x: x_1 \backslash h \backslash x \backslash \text{mod } p \backslash h \backslash x \backslash \text{mod } p \backslash \text{mod } p, p \geq N$. Similar to $\backslash \text{MOD}$ but uses a BST (proposed in this study)

The $\backslash \text{MAMD}$ and $\backslash \text{MOD}$ are theoretically nearly identical (see Supplementary Section 1). However, $\backslash \text{MAMD}$ uses a BST to lower the time complexity. Consider a window of hash values. Roughly stated, the $\backslash \text{MAMD}$ link function only needs four operations as we are sweeping the window over the sequence; find minimum element (no modulo wrap-around), find the closest element to a specific value (modulo wrap-around), add incoming element, and remove outgoing element. These operations can all be performed in logarithmic time with a BST. The $\backslash \text{MAMD}$ link function is described in detail in Supplementary Section 1. We will discuss the computational complexity of all methods in Section 2.6. In this section, we only discussed linking the first two strobos. Linking additional strobos can be done recursively by applying the same link function between the previous resulting randstrobe hash value b with the next candidate downstream strobos x_m , $m > 2$ as $\backslash b \backslash x_m \backslash$.

2.4.3 Sampling comparator

The comparator function, here denoted $c \backslash x \backslash$ specifies the criteria for which we select strobe x_1 among candidates x_1^i . To our knowledge, the only sampling comparator that has been proposed is $c_{\min} \backslash x: x_1^i \backslash \argmin_{x_1^i \in W} \backslash x: x_1^i \backslash$ (Sahlin 2021a, 2022, Xu et al. 2023), where W is the collection of strobos in the window defined by w_{\min} and w_{\max} . In this study, we propose $c_{\max} \backslash x: x_1^i \backslash \argmax_{x_1^i \in W} \backslash x: x_1^i \backslash$. The comparator can influence the result for some hash and link constructions as we will see in our benchmark.

2.4.4 The final seed hash value

We have so far discussed only how to select strobos. However, once the strobos have been decided, we need to represent the randstrobe with a *final hash value*. The final hash value is what should be indexed and queried, e.g. a seed-and-extend mapping framework. We denote the function to produce the final seed hash value as $f \backslash x: x_1 \backslash \dots \backslash x_n \backslash$. We need the function f to be as uncorrelated with the link function as possible. If we would use the hash value that comes out of $\backslash x: x_1 \backslash$ with, e.g. c_{\min} , we are projecting hash values to the minimum value in each window. This leads to unnecessary hash collisions compared to a uniform hash function. Furthermore, as mentioned in Sahlin (2021a), it is important to avoid symmetric functions $f \backslash x: x_1 \backslash \dots \backslash x_n \backslash$ (e.g. $f \backslash x: x_1 \backslash \dots \backslash x_n \backslash x_0 \backslash x_1 \backslash$) if distinguishing direction from, e.g. inversions is important [although a symmetric function is used to forward and reverse complements seeds in, e.g. read mapping (Sahlin 2022)]. Taking into consideration the above we use

$$f \backslash x: x_1 \backslash \dots \backslash x_{n-1} \backslash \begin{cases} 2x_0 - x_1 & \text{if } n \leq 2; \\ 2f \backslash x: x_1 \backslash \dots \backslash x_{n-2} \backslash - x_{n-1} & \text{if } n > 2; \end{cases}$$

This formulation allows f not to have any apparent correlation with any of the benchmarked link functions, as we will see in Section 3.

2.5 Linking more than two strobos

Generally, to link x_m to $x_0 \dots x_{m-1}$, $m \geq 2$, we use $\backslash b \backslash x_m \backslash$ where x_m are the candidate strobos in the window, and b denotes a *base value* calculated from the previous m strobos. We set the b equal to the previous strobos' final hash value, e.g. $b \backslash x: x_1 \backslash$ and $\backslash b \backslash x_2 \backslash$ in the case of three strobos. This method can be applied recursively.

2.6 Time complexity

Before discussing computational complexity, we make the following classifications of our link functions:

Cheap computation: This group includes $\backslash \text{MOD}$, $\backslash \text{AND}$, $\backslash \text{BC}$, $\backslash \text{XOR}$, and $\backslash \text{MAMD}$. We denote them as computationally cheap because the hashing and linking can be separated. That is, we only need to calculate hash values once for each strobe, and the link function can be applied after.

Expensive computation: This group includes $\backslash \text{CC}$, and $\backslash \text{XV}$. For these methods, we need to evaluate the hash value for the combination of x_0 and all its candidate downstream strobos, for each new x_0 .

The time complexity of constructing randstrobos from a string of length $|S|$ varies with the link-function class. Let t_h be the time complexity for the hash function, n the number of strobos, and $W \backslash w_{\max} \backslash w_{\min} \backslash$ be the window size. Then, $S \backslash nw_{\max} \backslash$ the number of randstrobos constructed from S . We assume that the linking operators (i.e. $\backslash \&$, $\backslash \%$, $\backslash \text{mod}$, $\backslash |$) can be performed in constant time, although the practical runtime varies among the operators with $\backslash \%$ being cheaper to perform while $\backslash |$ being relatively expensive.

Expensive computation methods perform $\backslash nW \backslash$ hash calculations, and nW other operations (such as $\backslash \&$, $\backslash \%$, $\backslash \text{mod}$, $\backslash |$), per randstrobe. So the total complexity is $O \backslash |S| \backslash nw_{\max} \backslash \backslash nW \backslash \backslash nW \backslash$. Cheap computation methods spend at most $\backslash |S| \backslash \backslash nW \backslash$ hash calculations and $\backslash |S| \backslash nw_{\max} \backslash \backslash nW \backslash$ on other operations, in total. So the total complexity is $O \backslash |S| \backslash \backslash nW \backslash \backslash |S| \backslash nw_{\max} \backslash \backslash nW \backslash$. If we assume that $|S| \backslash nw_{\max} \backslash$ and $t_h \backslash$ i.e. the complexity of t_h is at least a constant), we can simplify the expression of the time complexity of expensive computation methods and cheap computation methods to $O \backslash |S| \backslash nW \backslash$ and $O \backslash |S| \backslash \backslash nW \backslash$ respectively.

Lastly, the $\backslash \text{MAMD}$ link function is part of the cheap computation category. However, the time complexity is further reduced to $O \backslash |S| \backslash \backslash nW \backslash \log W \backslash$ through the logarithmic time complexity of searching for elements (see Supplementary Section 1 for details). While the BST implementation increases the constant coefficient through the BST overhead, we will see that the speed-up is substantial for large windows. We have abstracted over the exact time complexity of the hash functions. The cheapest computation is h_{NO} which only streams over the sequence without performing hashing. Some hash functions also support streaming (Mohamadi et al. 2016) and can lower t_h .

2.7 Evaluation metrics

There are different sampling biases that can arise as illustrated in Fig. 1. We were not able to find a singular metric that captured all of these biases, instead, we propose four suitable metrics that would capture cases B–E in Fig. 1. A desirable result is that the selection of the second (or any downstream) strobe is performed as uniformly in the window and as independently of the previous seed as possible. Several seed-based applications also require fast construction; therefore, we also benchmark construction runtime.

2.8 Notation for evaluation metrics

Let N be the total number of seeds constructed from a string S , and M the number of seeds with distinct final seed hash values in S . We let i and j be index variables over the set of randstrobes seeds sorted by their first strobe position. Since we here sample one randstrobe per position in S , the index variables are equivalent to the start position of the seed, and the N seeds can be ordered with respect to the start position on S . We let s_{jk} refer to the k th strobe in seed i and p_{jk} to its position in S .

2.9 E-hits

The E-hits metric was introduced in Sahlin (2022). It provides a number between 1 and $|S|$, which is the expected number of times a seed occurs in the reference. The E-hits metric was used as a measure for expected seed repetitiveness in S when sampling reads uniformly at random from a reference string S , assuming S is much larger than the span of the seed (Sahlin 2022). We restate the E-hits metric here for self-containment. Let $i \in \{1, \dots, M\}$ be an index variable over the set of distinct seeds in S and $N > M$ be the total number of seeds in S (multi-set). Let x_i denote the number of times seed i occurs in S . Let q_i be the probability of producing seed i when selecting a seed randomly from the N seeds. The E-hits metric is then the expected value over seed hits $E[X]$ computed as

$$E[X] = \sum_{i=1}^M q_i x_i \cdot \sum_{i=1}^M \frac{x_i}{N} \cdot \frac{1}{N} x_i^2. \quad (1)$$

In this study, seeds are represented as hash values. The above formula is equivalent if we replace the notion of a seed with the hash value representation of a seed. In this case, E-hits measure the expected number of identical hash values, which includes both repetitive seeds and non-desired hash collisions. We will measure the E-hits for the final seed hash values produced with f , and denote this quantity E_f . This is the same use of E-hits as in Sahlin (2022).

2.10 E-hits of inter-strobe distance and strobe position

The idea and formulation of E-hits can be used to measure the repetitiveness of other quantities. To measure strobe-distance clumping (bias B) and periodicity clumping (bias D) in Fig 1, we look at the distribution of inter-strobe distances within a randstrobe. Let d_{jk} be the distance between the first strobe and the k th strobe in seed j . We let x_i in Equation (1) be the number of times we observe distance d_{jk} . Equation (1) then measures the expected number of times we observe the distance d_{jk} when randomly drawing a seed from S . We denote this quantity as E_d and omit index variable k when it is clear from the context.

We measure second-strobe clumping (bias C) by computing the repetitiveness of the position of k th strobes in S . Let x_i in Equation (1) represent the number of times we observe the k th strobe selected at position p in S . Then, the E-hits formula measures the expected number of times position p was sampled as the k th strobe when drawing a seed uniformly at random from S . We denote this quantity as E_p (omitting index variable k when clear from context).

2.11 The conflict metric

To study complex dependencies (termed other clumping; Case E) as depicted in Fig. 1, we introduce the *conflict metric*, which aims to measure the size of the overlaps of strobes from a set of neighboring randstrobes with start positions in $[i, j]$, $i < j$. An overlap higher than what is expected under random sampling indicates selection bias. Let $o_{ij}^{(k)}$ be the number of overlapping positions of the k th strobe between two randstrobes i and j . Then $\sum_{k=1}^n o_{ij}^{(k)}$ is the total number of overlapping positions between two randstrobes. The conflict metric for randstrobe i is then defined as

$$C_i = \max_{j \in [i+1, i+m], \min_{k \in [1, n]} o_{ij}^{(k)} \geq 1} \sum_{k=1}^n o_{ij}^{(k)}$$

In other words, C_i is the largest observed overlap with any of the m consecutive downstream randstrobe seeds. We let the conflict metric (C) be the value of C_i averaged over all seeds in S . The above formula does not take into account that strobes of different orders (k) between neighboring randstrobes might overlap. However, even if this is possible for some values of w_{min} , it does not originate from the bias that we want to measure, and can therefore be omitted.

3 Results

We evaluated all compatible combinations of \sim , c , and h . Some combinations, such as h_{TW} with \sim_{CC} , are incompatible with strobes larger than 16 nucleotides (32 bits) because h_{TW} is designed for 64-bit integers. We use a simulated highly repetitive sequence (SIM), a set of 20 *Escherichia coli* genomes (E20), and the CHM13 human chromosome Y from the T2T assembly (Nurk et al. 2022) (ChrY) to evaluate pseudo randomness for randstrobes with $n \geq 2$. For runtime experiments, we used a simulated string of length 15 million. We also evaluated randstrobes $n \geq 3$ on the SIM dataset. Details of the experiment design and rationale are found in the Supplementary Section 2.

3.1 Pseudo-randomness

As for pseudo-randomness, we observed similar trends for the methods across the SIM, E20 and ChrY datasets. We also observed that the three hash functions (h_{WY} , h_{TW} , h_{XX}) had very similar results, we therefore focus on presenting the data for the SIM dataset using only h_{WY} compared to not hashing (h_{NO}) here. Results with all hash functions for SIM, E20, and ChrY are found in Supplementary Materials. Our benchmark highlights the following takeaways.

Hashing strobes: Always use a hash function to hash the strobes before linking (applicable to all link functions except \sim_{CC}), otherwise most link functions will be subject to the same form of severe bias (Fig. 2 and Supplementary Figs S1–S3).

Figure 2. Results for metrics E_d (upper panels), E_p (middle panels), and C (lower panels) for randstrobes with parameter settings $i_l=20$, $w_{min}=21$, $w_{max}=100$ for the repetitive sequence dataset. The x-axis shows the different linking methods, and the min and max comparators are shown in left and right panels, respectively. We have normalized the values with a near ideal result produced by simulating strobes uniformly at random in the window with $\text{rand}()$. Therefore, a value of 1.0 indicates best possible outcome (indicated by black dashed line).

Table 1. Overview of link functions and comparator functions based on the results from our experiments.^a

Category	Link function	Comparator	Introduced	Bias	Speed	Uniqueness	Comment (strength/weakness).
Expensive	CC	Any	This study ^b	—	Slow	High	Slow but supreme randomness.
	XV	Any	This study	—	Slow	High	Slow but supreme randomness.
	XOR	c_{min}	(Xu <i>et al.</i> 2023)	E_p, E_d, E_c	Fast	Low	XOR with c_{min} collapse similar regions leading to repetitiveness. Application determines if desired.
		c_{max}	This study	E_p	Fast	High	Fastest method with good randomness.
Cheap	MAMD	Any	This study	E_p	Fast ^c	High	Only method to scale for very large windows $i_l > 1000$.
	AND	Any	(Sahlin 2021a)	E_p	Fast	High	Fast but higher E_p than XOR .
	MOD	Any	(Sahlin 2021b)	E_p	Medium	High	Slower than XOR but not sensitive to the comparator.
	BC	c_{min}	(Sahlin 2022)	E_p, E_d, E_c	Slow	High	Designed to be biased.
		c_{max}	This study	E_p, E_d	Slow	High	sampling at the beginning of the window more often. As slow as expensive methods.

^a Results are described under the assumption that a hash function is used to hash the strobes (applicable to all link functions except CC).

^b Mentioned in Sahlin (2021a) but neither used nor studied.

^c Too much overhead to be used for small windows.

Link function: The two expensive methods (XV , CC) achieve the best pseudo-randomness (Fig. 2 and Supplementary Figs S1–S3). As for the computationally cheap methods, different methods have different bias (Table 1).

Comparator: Comparator choice is only important for some link functions. Cheap computation XOR-based methods XOR and BC exhibit high bias with the c_{min} comparator. This is because the c_{min} comparator will select a candidate strobe to be identical to the previous strobe if present in the window (XOR value of 0) while c_{max} will have the opposite behavior. Since our repeats in the SIM dataset have reoccurring distances between them (which also happens in biological sequences), it causes distance clumping (bias B) and negative positional clumping (bias C).

3.2 Seed repetitiveness

Seed repetitiveness in the reference is crucial for applications such as read mapping (Sahlin 2022, Ekim *et al.* 2023, Maier and Sahlin 2023, Shaw and Yu 2023). We use k -mers of length 40 nt, corresponding to the same number of sampled positions in the randstrobes, as a reference method in this benchmark. The k -mers are stored as two strobes with the same final function as the randstrobes, $f(i_l, x_1, \dots, x_{i_l}) = 2x_0 \oplus \dots \oplus x_{i_l}$. We first verified that using our final hash function f for seed representation resulted in minimal hash collisions (Supplementary Fig. S4). Since hash collisions were not significant, we computed the E-hits of the final seed hash value (E_f), for all methods. As with randomness, it is important to use a hash function before linking strobes (Fig. 3 and Supplementary Figs S1–S3). Additionally, we observed that

randstrobes generally have lower E_f than k-mers for most hash and link functions, but repetitiveness can increase with specific combinations (Fig. 3).

3.3 Runtime performance

Figure 4 shows the construction time for window sizes using $w_{max} = 100$ and $w_{max} = 1000$, respectively. Expensive computation methods ($_CC$ and $_XV$) are performing a factor of

nW more hash computations. However, they are only about 2.5–4 times slower than the average cheap computation methods when using h_{WY} as hash function (Fig. 4). One explanation could be cache efficiency. We also observe that the $_BC$ and $_MOD$ are substantially slower than other methods in the cheap-computation class. Finally, when constructing randstrobes with large windows, $_MAMD$ is much faster than other methods (Fig. 4, lower panels). This is due to the BST

Figure 3. Normalized E-hits of seed hash values for various to construct randstrobes with parameters $n = 2$, $l = 20$, $w_{min} = 21$, $w_{max} = 100$ compared to k-mers of size 40. Lower value is better.

Figure 4. Runtime (seconds) on 45 instances for each combination on a 15 million nt simulated string. Each combination generates randstrobes with $n = 2$, $l = 20$, $w_{min} = 21$, and $w_{max} = 100$ (upper panel) and $w_{max} = 1000$ (lower panel).

implementation instead of a linear search across each window. However, due to its special updating technique utilizing arithmetic properties of the modulo operator, the method can only be used with the modulo link function. As for the hash functions, h_{WY} performs better than h_{XX} and h_{TW} on our data for the expensive computation class, where strobos are represented by a struct of two 64-bit integer strobos.

3.4 Randstrobos in large windows

The \backslash_{MAMD} link function enables efficient construction of randstrobos in large windows. We were interested in the uniqueness of seeds that \backslash_{MAMD} produced compared to one of the best-performing methods \backslash_{CC} (using C_{max}). We used $p \approx 100:001$ in the previous analysis. For this analysis, we set $p \approx 19:019; 684:767; 739:993$. The value of p needs to be significantly larger than the window size but smaller than the maximum hash value to guarantee high pseudo-randomness. To our knowledge, the value of p has no specific influence beyond that. We investigated the expected uniqueness (E-Hits) of the seeds computed across chromosome Y of the CHM13 assembly (Fig. 5, left panel). In the figure, a window size of 0 corresponds to k -mers of size 256. We make two key observations about the uniqueness of seeds. First, we note that there is no substantial difference between the two link functions on chromosome Y from the CHM13 assembly, including telomere regions and many repetitive multigene families. Second, we observe that the E-hits function is not linearly decreasing, which we initially expected. Minimum repetitiveness occurs at $w_{max} \approx 2:000$ instead of the largest evaluated window at $w_{max} \approx 10:000$. This is likely explained by the observation that, beyond a certain window size, the more likely it is that the same pair of strobos is found and linked. We also looked at how the runtime scaled with window size. Figure 5 (right panel) shows the median runtime from 10 runs on the *E.coli* genome of 5.5 million nucleotides. Our BST implementation greatly outperforms \backslash_{CC} .

3.5 Implementing C_{max} in strobealign

Strobealign is a read mapper that use randstrobos created from syncmers (Edgar 2021) using C_{min} together with \backslash_{BC} , which we observed were particularly bad in terms of seed uniqueness and randomness (Figs 2 and 3). Guided by our benchmark, we wanted to investigate whether C_{max} would result in better mapping results. The experiment is described in

detail in Supplementary Section 4. We did not observe a direct improvement in strobealign's accuracy when run with C_{max} compared to the default version that uses C_{min} (Supplementary Tables S1 and S2). However, we observed a large improvement in accuracy for the shorter read lengths when combining the results of the two runs of strobealign (details in Supplementary Section 4).

4 Discussion and conclusions

Constructing randstrobos involves four modular operations: computing individual strobe hash values (hash function), determining hash values for linked strobos (link function), selecting the final randstrobe from multiple candidates using a comparator function, and computing the hash value for the chosen randstrobe. The initial three operations (hash, link, and comparator functions) yield diverse results based on the combination of functions used. Our study introduced and benchmarked both novel and previously used hash, link, and comparator methods for randstrobe construction, accompanied by metrics to evaluate method biases. Our benchmark revealed biases in existing techniques and can offer general guidance for which methods to use when utilizing randstrobos as sequence comparison seeds. From our evaluation, we conclude the following.

Hashing: Always hash the strobos before linking with a computationally cheap link method. It does not result in a large overhead in construction time (Fig. 4) while being beneficial for pseudo-randomness (Figs 2 and 3). The hash functions have roughly the same pseudo-randomness performance, but the h_{WY} function had the best runtime. A downside with hashing compared to the 2-bit encoding is that nucleotide level information of the seed is lost. This should be factored into the decision for the application at hand.

Linking: In short, we believe \backslash_{CC} or \backslash_{XV} should be used when highest pseudo-randomness is desired, \backslash_{XOR} (with C_{max}) should be used when speed is important, and \backslash_{MAMD} for use cases with very large windows (Table 1). We do not see any benefit with using \backslash_{AND} and \backslash_{MOD} over \backslash_{XOR} . Finally, \backslash_{BC} is a special function designed for when biased sampling is desired, as in Sahlin (2022).

Figure 5. A comparison between \backslash_{MAMD} and \backslash_{CC} with parameters $i \approx 128$; $w_{min} \approx 129$; $w_{max} \approx x$ where x is plotted on the x-axis. Left panel shows E-hits on Chromosome Y from the CHM13 human assembly (Nurk *et al.* 2022). The right panel shows median runtime out of 10 runs on an *E.coli* genome of 5.5 million nucleotides.

Comparator: The comparator matters for some link functions (Table 1). For example, an XOR-based link-function projects identical hash values to 0. Therefore, a min comparator will select identical strobes if present, while a max comparator will be inclined to select differing strobes. Consequently, in repetitive regions with occasional variations (e.g. SIM dataset) where the window is larger than the repeat length, the min comparator will tend to collapse seeds while a max comparator has the opposite behavior. This however implies that in such regions, the max comparator will be less robust to sequencing errors in reads. These two effects pull in different directions when it comes to read mapping. We observed no substantial difference between them in strobealign (Supplementary Tables S1 and S2) but combining their results led to large improvement for shorter reads (Supplementary Tables S1 and S2).

Final seed hash value function: Choose a final seed hash value function that is uncorrelated to the link function to avoid hash collisions. For example, we used $2x_0 - x_1$ for two strobes that did not show any apparent correlation with the link functions we benchmarked (Supplementary Fig. S4).

5 Future work

Efficiently applying hash and link functions can benefit cheap computation methods. A rolling hash function, like ntHash (Mohamadi *et al.* 2016), can enhance hash computation in these methods. This optimization proves valuable when hashing is relatively more expensive than linking, particularly for larger window sizes. Additionally, a link function \sim_{MAMD} was designed using arithmetic reasoning to reduce construction time complexity. Further investigation is needed to determine if the rolling hash approach allows for arithmetic operations permitting efficient linking methods.

We observed improved accuracy when combining results from min and max comparators in strobealign. Our proof-of-concept approach involved running strobealign twice and post-processing the alignments, resulting in slightly more than twice the runtime compared to a single run. To mitigate an increase in runtime, integrating seeds from both comparators into strobealign is a solution. This increases memory usage but may not affect runtime since costly rescue-alignment calls may be lowered due to fewer regions without seed matches.

Acknowledgements

We thank Daniel Liu and Heng Li for useful feedback on the linking methods.

Supplementary data

Supplementary data are available at *Bioinformatics* online.

Conflict of interest

R.P. is a co-founder of Ocean Genomics Inc.

Funding

Kristoffer Sahlin was supported by the Swedish Research Council (SRC, Vetenskapsrådet) [2021-04000]. Marcel Martin was financially supported by the Knut and Alice

Wallenberg Foundation as part of the National Bioinformatics Infrastructure Sweden at SciLifeLab. Mengyang Xu was supported by National Natural Science Foundation of China [32100514]. Giulio Ermanno Pibiri was partially supported by DAIS—Ca' Foscari University of Venice within the IRIDE program.

Data availability

The human Y chromosome from the CHM13 genome was downloaded from <https://github.com/marbl/CHM13?tab=readme-ov-file#downloads> and the 20 E. coli strains used in the E20 benchmark is found in supplementary materials and can be downloaded from RefSeq (<https://www.ncbi.nlm.nih.gov/refseq/>). The code for generating the simulated dataset is available at <https://github.com/Moein-Karami/RandStrobes>.

References

- Alser M, Rotman J, Deshpande D *et al.* Technology dictates algorithms: recent developments in read alignment. *Genome Biol* 2021;**22**:249.
- Chin C-S, Khalak A. Human genome assembly in 100 minutes. *bioRxiv*, <https://doi.org/10.1101/705616>, 2019, preprint: not peer reviewed.
- Edgar R. Syncmers are more sensitive than minimizers for selecting conserved k-mers in biological sequences. *PeerJ* 2021;**9**:e10805. <https://doi.org/10.7717/peerj.10805>.
- Ekim B, Berger B, Chikhi R. Minimizer-space de Bruijn graphs: whole-genome assembly of long reads in minutes on a personal computer. *Cell Syst* 2021;**12**:958–68.e6.
- Ekim B, Sahlin K, Medvedev P *et al.* Efficient mapping of accurate long reads in minimizer space with mapquik. *Genome Res* 2023;**33**:1188–97. <https://doi.org/10.1101/gr.277679.123>.
- Fan J, Khan J, Singh NP *et al.* Fulgor: a fast and compact k-mer index for large-scale matching and color queries. *Algorithms Mol Biol* 2024;**19**:1–21.
- Firtina C, Park J, Alser M *et al.* Blend: a fast, memory-efficient and accurate mechanism to find fuzzy seed matches in genome analysis. *NAR Genom Bioinform* 2023;**5**:lqad004.
- Li H. Minimap2: pairwise alignment for nucleotide sequences. *Bioinformatics* 2018;**34**:3094–100.
- Ma B, Tromp J, Li M. PatternHunter: faster and more sensitive homology search. *Bioinformatics* 2002;**18**:440–5. <https://doi.org/10.1093/bioinformatics/18.3.440>.
- Maier BD, Sahlin K. Entropy predicts sensitivity of pseudo-random seeds. *Genome Res* 2023;**33**:1162–74. <https://doi.org/10.1101/gr.277645.123>.
- Marchet C, Boucher C, Puglisi SJ *et al.* Data structures based on k-mers for querying large collections of sequencing data sets. *Genome Res* 2021;**31**:1–12. <https://doi.org/10.1101/gr.260604.119>.
- Mohamadi H, Chu J, Vandervalk BP *et al.* ntHash: recursive nucleotide hashing. *Bioinformatics* 2016;**32**:3492–4.
- Nip KM, Hafezqorani S, Galalova KK *et al.* Reference-free assembly of long-read transcriptome sequencing data with rna-bloom2. *Nat Commun* 2023;**14**:2940.
- Nurk S, Koren S, Rhie A, *et al.* The complete sequence of a human genome. *Science* 2022;**376**:44–53.
- Roberts M, Hayes W, Hunt BR *et al.* Reducing storage requirements for biological sequence comparison. *Bioinformatics* 2004;**20**:3363–9. <https://doi.org/10.1093/bioinformatics/bth408>.
- Sahlin K. Effective sequence similarity detection with strobemers. *Genome Res* 2021a;**31**:2080–94. <https://doi.org/10.1101/gr.275648.121>.
- Sahlin K. Strobemers: an alternative to k-mers for sequence comparison. *bioRxiv*, 2021b, preprint: not peer reviewed.
- Sahlin K. Strobealign: flexible seed size enables ultra-fast and accurate read alignment. *Genome Biol* 2022;**23**:260.
- Sahlin K, Medvedev P. Error correction enables use of Oxford Nanopore technology for reference-free transcriptome analysis.

- Nat Commun* 2021;**12**:2. <https://doi.org/10.1038/s41467-020-20340-8>.
- Sahlin K, Baudeau T, Cazaux B *et al.* A survey of mapping algorithms in the long-reads era. *Genome Biol* 2023;**24**:133.
- Shaw J, Yu YW. Proving sequence aligners can guarantee accuracy in almost $o(m \log n)$ time through an average-case analysis of the seed-chain-extend heuristic. *Genome Res* 2023;**33**:1175–87. <https://doi.org/10.1101/gr.277637.122>.
- Xu M, Guo L, Qi Y *et al.* Symbiont-screener: a reference-free tool to separate host sequences from symbionts for error-prone long reads. *Front Mar Sci* 2023;**10**. <https://doi.org/10.3389/fmars.2023.1087447>.