An evolutionary approach to predict the orientation of CRISPR arrays

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CRISPR-Cas is a defense system of bacteria and archaea against phages. Parts of the foreign DNA, called spacers, are incorporated into the CRISPR array which constitutes the immune memory. The orientation of CRISPR arrays is crucial for analyzing and understanding the functionality of CRISPR systems and their targets. Several methods have been developed to identify the orientation of a CRISPR array. To predict the orientation, different methods use different features such as the repeat sequences between the spacers, the location of the leader sequence, the Cas genes, or PAMs. However, those features are often not sufficient to predict the orientation with certainty, or different methods disagree.

Remarkably, almost all CRISPR systems have been found to insert spacers in a polarized manner at the leader end of the array. We introduce *CRISPR-evOr*, a method that leverages the resulting patterns to predict the acquisition orientation for (a group of) CRISPR arrays by reconstructing and comparing the likelihood of their evolutionary history with respect to both possible acquisition orientations. The new method is independent of Cas type, leader existence and location, and transcription orientation. *CRISPR-evOr* is thus particularly useful for arrays that other CRISPR orientation tools cannot predict confidently and to verify or resolve conflicting predictions from existing tools. *CRISPR-evOr* currently confidently predicts the orientation of 28.3% of the arrays in the considered subset of CRISPRCasdb, which other tools like CRISPRDirection and CRISPRstrand cannot reliably orient. As our tool leverages evolutionary information we expect this percentage to grow in the future when more closely related arrays will be available. Additionally, *CRISPR-evOr* provides confident decisions for rare subtypes of CRISPR arrays, where knowledge about repeats and leaders and their orientation is limited.

CRISPR-Cas | bacterial genomics | spacer array | spacer gain and loss | likelihood functions | microbiomes | CRISPR orientation

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Introduction

CRISPR-Cas systems are powerful defense systems of bacteria and archaea and provide adaptive immunity against foreign elements like phages and plasmids [1, 2]. One component of the system are the so called CRISPR associated (Cas) genes that allow the bacterium to produce protein complexes that are able to interact with mobile genetic elements. Depending on the composition of Cas genes CRISPR-Cas systems are classified into a multitude of classes and types by the Cas genes found in the genome and the presence of CRISPR arrays [3, 4]. The other component is the CRISPR array and composed of repeats which are interspaced by small DNA snippets called spacers. These spacers are expressed as crRNA which fit into Cas protein complexes to target specific foreign elements and destroy or block them [1, 2].

The systems adapt to new threats by acquiring new spacers from snippets of invader sequences which are then inserted in the CRISPR array with a new repeat unit [2, 5]. Notably, new spacers are almost always inserted at the so called leader end [5, 6]. The leader is a non-coding sequence of variable length (up to a few hundred bp) with limited sequence conservation that is located, in general, at the 5' end of the array. The leader is responsible for regulating the acquisition and transcription of spacers [7–10].

CRISPR arrays evolve over time by the acquisition and deletion of spacers. Moreover, due to the polarized insertion process, spacer arrays constrain the evolutionary history as they are ordered by the time of their acquisition.

Importance of orientation for CRISPR systems. DNA and RNA are both transcribed and synthesized in the 5'-3' direction. Thus, the orientation of CRISPR arrays carries importance in a multitude of facets, including the processing of spacers into crRNA, the interference and adaptation mechanisms and thus the understanding of different Cas types. In particular, it is essential to identify leader regions, to identify transcription initiation and termination regions, to determine the orientation of protospacers and to identify PAM sequences [9, 11, 12]. Furthermore, inserting foreign arrays into new organisms in the wrong orientation may cause them to be mischaracterized as non-functional in experiments.

The orientation is also of crucial importance when investigating the ecology and evolution of CRISPR arrays. The acquisition process of most CRISPR arrays was found to be polarized [1, 2]. Thus, if the orientation prediction is wrong the whole ordering would be interpreted wrongly and evolutionary inference is likely to produce false results [13].

Orientation concepts and tools for CRISPR-Cas systems. In the following, we introduce the concepts and the corresponding tools to predict the CRISPR array orientation. Tools can provide differing predictions for the orientation of

CRISPR arrays due to different orientation concepts, but also between the predictions of tools using the same concept. We give a short overview of the different concepts in Table 1.

The *transcriptome* can be used to infer the orientation of arrays [14], however, this information is typically not available. We thus focus on other available concepts and tools here.

In most known CRISPR arrays found in nature, there are Cas genes in close proximity. Thus, Milicevic et al. suggest to predict the CRISPR orientation based on the *Cas-orientation*. The Cas-orientation is determined by the transcription orientation of the Cas genes close to a respective CRISPR array (< 1000 bp distance) [15].

Furthermore, CRISPR arrays are highly characterized by their *leader*, which is found at only one end of the array. Typically, the leader is found at the 5' end. Thus, one can determine the "leader-orientation" by determining the location of the leader.

A closely related orientation concept is based on the *repeats*. The "repeat-orientation" is characterized by mutation patterns along the array. Due to the molecular functionality of the (polarized!) insertion and deletion process of spacers, repeats degrade in 5'-to-3' direction by accumulating mutations [16], moreover, the mutations are concentrated at the ends of repeats [17].

Both the leader- and repeat-orientation are closely related concepts and one of the most commonly used prediction tools for CRISPR arrays, CRISPRCasFinder [18], relies on both leader- and repeat based orientation methods. More precisely, CRISPRCasFinder employs a combination of leader- and repeat-orientation [18] and incorporates "CRISPRDirection" [16]. The orientation is predicted by, firstly, trying to find the leader by searching for relative AT richness at both ends of the array and comparing the distance to the closest coding genes at both ends. Secondly, CRISPRDirection considers specific sequence motifs and mutations, the RNA secondary structure, AT content in the repeats, and degeneracy in the 3' end of the array [18, 19].

"CRISPRidentify" [20] is another commonly used tool to find CRISPR systems that relies on repeat based orientation by incorporating "CRISPRstrand" [17]. CRISPRidentify does not use any additional information about the leader for the orientation prediction, but CRISPRleader within CRISPRidentify can annotate the leader [9] by using the repeat-orientation predicted by CRISPRstrand.

CRISPRstrand uses domain expert knowledge about repeats to identify important features. It was observed that it is useful to partition the consensus repeat into different blocks as the mutation pattern along the consensus repeat strongly diverges. CRISPRstrand encodes the consensus repeats as graphs, which additionally, contain information about mutations and their position in the repeat. Then they train a graph kernel model on the encoded graphs of a curated dataset [17].

Vink et al. identified the orientation of many CRISPR arrays by identifying the respective protospacers of the spacers in the array and *locating the protospacer adjacent motifs* (PAM) [11]. PAMs are (generally) 3 nucleotide long sequences at one side of the protospacer. They are used by the CRISPR-Cas system to correctly distinguish between spacers and protospacers to avoid autoimmunity. These sequences are quite specific to the systems Cas-(sub)type.

While all of these concepts are viable ways to predict the orientation, they not necessarily agree and do not always provide a reliable basis for a confident prediction. For example, for some CRISPR arrays Cas genes will be reversed or no Cas genes are located closely to the array, a leader sequence does not exist [9], no protospacers can be found, or the CRISPR arrays are too short to analyze the repeats. Consequently, novel concepts and methods for array orientation are necessary to expand the range of confidently orientable arrays.

Acquisition-orientation as a holistic evolutionary approach to predict array orientation. In contrast to the features used by previous approaches of orientation prediction, one (almost) universal property of CRISPR array evolution is the polarized acquisition of new spacers into the array. In most cases, acquisition is facilitated at the leader end, i.e. the 5' end [5, 6]. These polarized insertions lead to highly distinctive evolutionary patterns in the reconstructed ancestral history of groups of closely related CRISPR arrays.

We propose *CRISPR-evOr* a method that identifies what we call the "acquisition-orientation", the most likely end of acquisition. This orientation concept is closely related to the other orientation concepts, in particular, the leader- and repeat-orientation. However, instead of relying on markers in single CRISPR arrays or genomes, *CRISPR-evOr* uses a holistic approach that relies on reconstructions of the evolution of a group of CRISPR arrays. In the simple heuristic, we reconstruct the ancestral history with *SpacerPlacer* [13] two times. First, the evolution is reconstructed for the arrays in the given orientation. Then we reverse the CRISPR array spacer sequence and compute a second reconstruction. Subsequently, we compare the likelihoods of each reconstruction provided by *SpacerPlacer* to predict the most likely acquisition-orientation. In this way, we acquire an orientation prediction by consensus decision of all CRISPR arrays in the group. Figure 1 shows an example with a high confidence prediction where, clearly, the acquisition-orientation shown in a) can be favored over the reverse as shown in b), simply due to the much smaller number of evolutionary events.

We apply *CRISPR-evOr* on a large database of CRISPR arrays extracted from CRISPRCasdb [21]. We find that *CRISPR-evOr* is very decisive and allows us to predict orientations for arrays where the aforementioned orientation methods have low confidence. Thus, our tool increases the number of arrays where orientations can be reliably estimated.

Furthermore, on data where orientation predictions are already available, *CRISPR-evOr* helps to either reinforce the predictions, or resolve contradicting predictions of other tools, and thereby uncovers discrepancies among different orientation concepts dependent on the Cas type. We find that among the array subsets where multiple tools are confident, predictions by *CRISPR-evOr* almost always agree with other tools for two of the most abundant Cas types (I-E and I-F) in CRISPRCasdb. In particular for

types I-B and II-A, *CRISPR-evOr* agrees more often with CRISPRstrand than CRISPRDirection. The differences indicate that CRISPRDirection is well-attuned only for arrays of some Cas types. Moreover, *CRISPR-evOr*, PAM-orientation, CRISPRDirection, and CRISPRstrand often disagree with each other for type II-C, which hints at differences between acquisition-orientation and leader-, repeat- and PAM-orientation for type II-C.

Methods

Database and available orientation predictions. We rely on the CRISPRCasdb dataset collected by Fehrenbach et al. [13]. It is based on high-confidence arrays extracted from CRISPRCasdb [21] in July 2022. For each array we extracted the orientation prediction by CRISPRDirection made available in their database and applied CRISPRstrand to obtain the respective orientation predictions. These arrays were grouped successively by repeat, genus, Cas type to yield a large comprehensive dataset. We clustered spacers as described in [13] and removed groups which did not contain any spacer overlap between arrays or groups with completely the same arrays. Thus, each group contains only arrays of the same consensus repeat, genus and Cas type and are closely related in spacer content and we obtain a database of 518 groups with 5934 arrays. A breakdown of the Cas types in the dataset is shown in Supplementary Fig. S1. Note that [13] investigate and estimate parameters of the deletion process on a subset (334 groups, 4565 arrays with at least one deletion in the reconstruction) of the larger dataset used here.

Additionally, we used an updated version of the dataset collected by Vink et al., who extracted their dataset from CRISPRCasdb in February 2020 [11]. The updated version uses the same dataset of spacers, but contains more confident orientation predictions. The old version is available in their supplementary material. The database contains 4266 bacterial and archaeal genomes and a total of 221,591 spacers and their respective consensus repeat sequences. They subsequently matched these spacers against the NCBI nucleotide database and large metagenomic databases to obtain target matches. They then investigated the flanks of the matches to find the associated PAM sequences and predict the orientation of spacers based on the location of the respective PAM at the matching target's flanks. We then matched the repeats and respective spacers against our dataset to allow us to compare the orientation predictions. Thus, the dataset used for the evaluation of PAM-orientation versus *CRISPR-evOr* is the intersection between the dataset from [13] and Vink et al.'s dataset, both of which are part subsets of CRISPRCasdb. Furthermore, only 38.9% (2309) of the arrays and 77.2% (400) of the groups in Fehrenbach et al.'s dataset have PAM-orientation predictions.

Ancestral reconstruction with *SpacerPlacer*. The backbone of *CRISPR-evOr* is the ancestral reconstruction algorithm for groups of closely related CRISPR spacer arrays *SpacerPlacer* [13].

To prepare the spacer data, *SpacerPlacer* labels each unique spacer found in the dataset with a number and aligns the labeled spacer arrays with MAFFT [22]. This results in an Multiple Spacer Array Alignment (MSAA) where each column corresponds to a unique spacer and each row to a CRISPR array. Assuming a direction of acquisition yields a clear timeline of acquisition for each array, where the spacer near the acquisition end is the youngest spacer while the spacer further removed from the acquisition end are older spacers. *SpacerPlacer* constructs a Partial Spacer Insertion Order (PSIO) from all CRISPR arrays by combining all individual insertion orders together.

The reconstruction proceeds in two steps. In the first step *SpacerPlacer* computes a guide reconstruction based on a straightforward model allowing for independent deletions and insertions at each spacer site. The reconstruction is performed with a joint maximum likelihood approach proposed by Pupko et al. [23]. Naturally, as insertions can occur within the array, the reconstruction will not necessarily follow the PSIO, i.e. spacers can be inserted behind existing spacers.

In the second step *SpacerPlacer* refines the guide reconstruction by enforcing the pre-computed PSIO. The PSIO is enforced by finding contradictions, i.e. insertion events that are behind a leading spacer, along the tree. Each of these contradicting spacers is then fixed to be present at its respective parent node. Then, recomputing the reconstruction with these fixed states yields a new reconstruction, where some of the contradictions might be resolved. This process is repeated until all contradictions are resolved and results in a reconstruction that respects the PSIO. This process always finds a reconstruction without contradictions, where, in the worst case, most or all contradicting spacers end up being acquired at the root.

After both steps, we obtain an ancestral reconstruction that can be analyzed and used to estimate deletion model parameters. Furthermore, the reconstruction is visualized by rendering the respective tree with reconstructed acquisition and deletion events and the MSAA. For an example see Figure 1.

Computation of likelihoods with *SpacerPlacer*. *SpacerPlacer* is able to compute the likelihoods of reconstructions according to their proposed block deletion model (BDM), which they found to be a suitable model for spacer deletions in statistical analysis on the CRISPRCasdb dataset [13].

We use the simplified likelihood function and default parameters computed on the CRISPRCasdb dataset, as is recommended [13].

Tree estimation. We estimated trees based on the single-copy core genes of the corresponding sample for each group as is stated in [13]. We used Bakta to annotate genomes [25] and then the pangenome analysis tool panX [26] and IQ-TREE [27] to reconstruct trees. We used these core genome trees for all experiments and figures unless otherwise stated.

If no tree is provided by the user, *CRISPR-evOr* uses the provided functionality of *SpacerPlacer* to estimate trees based on the CRISPR arrays. In that case, *CRISPR-evOr* estimates the tree for each orientation , i.e. reconstruction run,

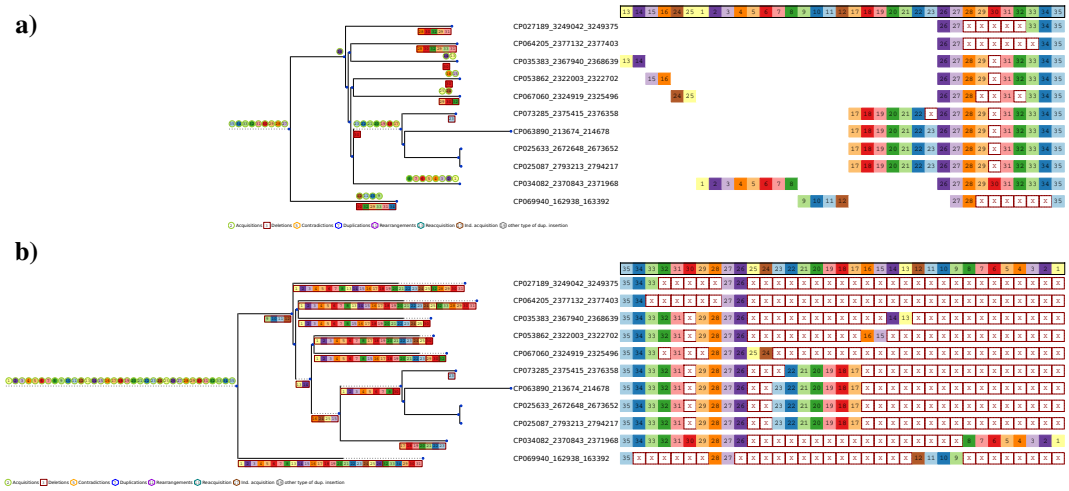


Fig. 1. Example of a group of arrays with high confidence orientation prediction. a) and **b)** show reconstructions of the same alignment of spacer arrays. b) has the orientation provided by CRISPRCasdb, while a) is simply the reconstruction of the same arrays but with reversed sequencing, i.e. reversed alignment. Note, that in b) all spacers are reconstructed to be inserted (green) at the root and thus the exceptional amount of deletion events (red) along the tree (also shown in red in the spacer alignment). The insertions are produced through the first spacer 54 (light blue) which is present at the root, as it is present in all arrays, and "pulls up" all other spacer insertions through the spacer insertion order. In contrast, in a) the insertions are distributed more evenly along the tree and thus the reconstruction avoids a lot of deletions. Comparing both reconstructions clearly indicates that the orientation of a) is more likely under the assumption of polarized insertions. The shown example is a group of *Klebsiella pneumoniae* (Cas type I-E) from CRISPRCasdb.

Orientation concept	Description and Tools/Papers using the concept
acquisition	Find the end of the array where new spacers are inserted by analyzing the evolution of groups of CRISPR arrays. **Tool:** *CRISPR-evOr* (new)
Cas	Determine the transcription orientation of Cas genes close to the array and identify their orientation with the array orientation. **Reference:** Milicevic et al. [15]
PAM	Find PAMs of CRISPR systems and locate the PAMs on matched targets of spacers to determine the orientation. **Reference:** Vink et al. [11]
repeat	Characterize the repeats according to available biological knowledge and their relation to transcription orientation. In particular, analyze the mutation pattern of the repeats along the length of the array. **Tools:** CRISPRstrand [17], CRISPRidentify [20], CRISPRmap [24]
leader + repeat	Use empirically measured characteristics to analyze the repeats and locate the leader sequence by analyzing the regions adjacent to the CRISPR array. These characteristics are weighted and combined to generate a prediction. **Tools:** CRISPRDirection [16], CRISPRCasFinder [18], CRISPRDetect [19]
transcriptome	Predict orientation by employing transcriptome sequencing to find the transcription orientation of arrays. **Tool:** TOP [14]

Table 1. Orientation concepts overview. We show all orientation concepts in use for CRISPR arrays that are referenced within this work. We give them labels to simplify referencing the different orientation concepts. Below the descriptions, we give reference papers which introduce and use the concepts and mention the tools that rely on the respective method for their orientation prediction.

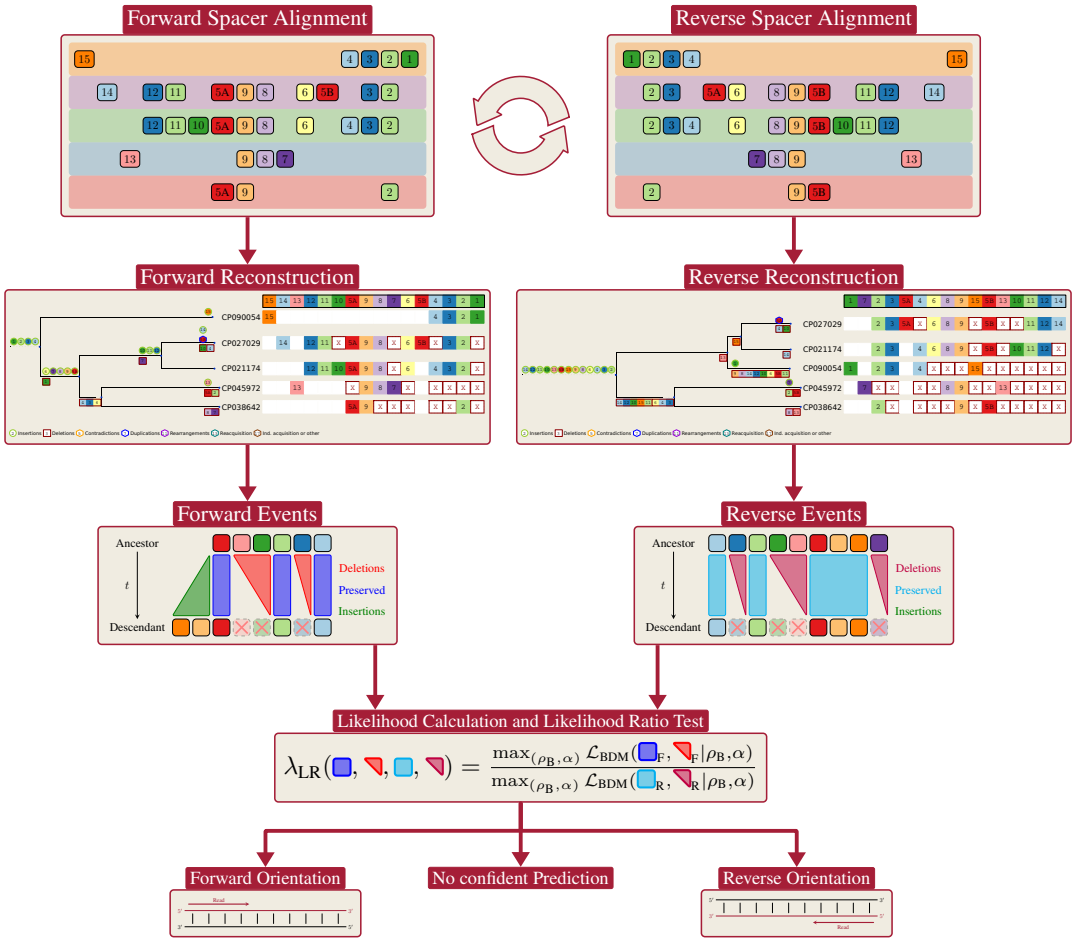


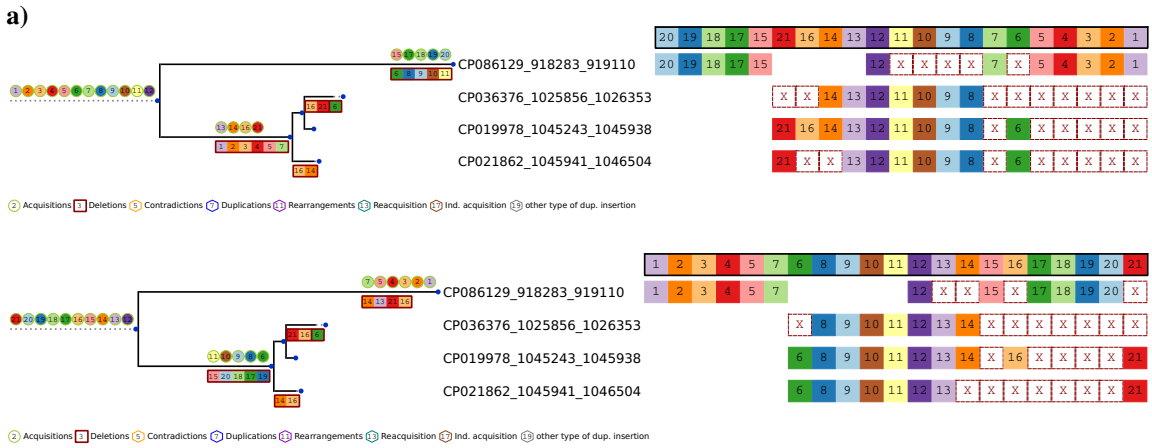
Fig. 2. *CRISPR-evOr* workflow. We show the steps taken by *CRISPR-evOr* to arrive at a prediction for a given group of spacer alignments. The left and right column show the same steps of reconstructing the ancestral history of a spacer alignment with *SpacerPlacer*. The differ only by their orientation, i.e. the alignments are turned around compared to each other. Note, that *SpacerPlacer* is heavily reliant on the partial spacer insertion order (PSIO) and thus, this difference can have major impact on the reconstruction. Furthermore, in this case here, no tree was provided to *SpacerPlacer* and thus, *SpacerPlacer* estimates a tree based on the spacer arrays for each orientation. After both reconstructions are performed, *SpacerPlacer* obtains maximum likelihoods of the respective reconstructions based on the reconstructed events. Then by comparing the ratio of both likelihoods, *CRISPR-evOr* decides for one of the orientations or, if the likelihoods are too close, determines that no confident prediction can be made.

separately since the trees themselves depend on the acquisition orientation and might differ substantially.
SpacerPlacer estimates trees using neighbor joining (NJ) or unweighted pair group method with arithmetic mean (UPGMA) with a simple distance function, which estimates the evolutionary distances between a pair of arrays assuming a polarized insertion model with blockwise deletions and suitable empirically estimated rates.

Simulations. Simulations were performed according to the methods described in [13]. First, a spacer array with length distributed according to the (rough approximation) of the stationary distribution of the BDM is sampled. Subsequently, starting at the root of a given tree, the array is evolved along the tree branches by sampling acquisition and deletion events. The acquisitions are polarized and the deletion events follow the BDM.
To evaluate the performance of *CRISPR-evOr* we simulated two datasets:
Dataset A: Here, Phylogenetic trees were sampled according to the coalescent and we simulated datasets with reasonable parameter values as described in [13]. Dataset B: For a more realistic setting, we used the core-genome based trees estimated in [13] for the CRISPRCasdb dataset (see tree estimation) and simulated datasets using their respective estimated evolutionary parameters. Note that the parameters estimated by [13] were estimated on groups with at least one observed deletion events, and thus all groups in the simulations have non-zero acquisition and deletion rates.
More information and precise numbers can be found in Supplementary Note 1A. Additionally, information about the simulation process can be found in the respective section and Supplementary Note 1 of [13].

Results

Orientation algorithm. We now describe the process with which *CRISPR-evOr* predicts the acquisition-orientation. Previous methods of estimating the strand orientation mostly rely on properties of the repeats and neighboring DNA



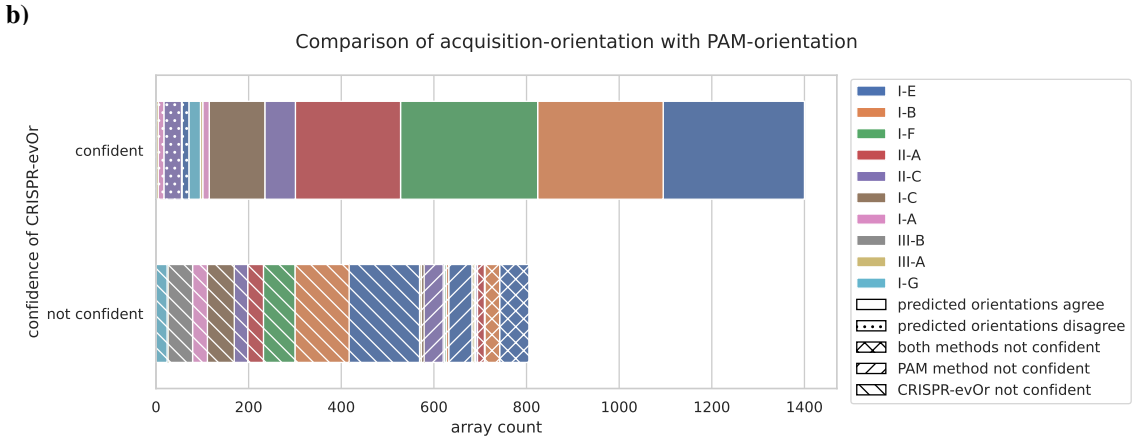


Fig. 3. Example of a group with low confidence orientation prediction and comparison between acquisition- and PAM-orientation. a) shows an example where *CRISPR-evOr* is not confident. Both orientations lead to reasonable reconstructions and although *CRISPR-evOr* is able to give a suggestion ($|L_{\text{ratio}}| = 1.51$ in favor of the lower reconstruction), it will not be confident according to our confidence threshold. The shown example is a group of *Streptococcus agalactiae* (Cas type I-C) from CRISPRCasdb. **b)** compares predictions between the PAM-orientation and *CRISPR-evOr* broken down according to Cas type. Only subtypes were a reasonable amount of data is available, i.e. at least 5 groups with more than 50 arrays, are shown. While there are many arrays were both methods can not make confident decisions, in cases where they are confident, they almost always agree. The only type that deviates from this behavior is type II-C.

sequence within a *single* genome. We, however, use spacer arrays of a *collection* of samples and their spacer acquisition process, which is known to be polarized, to estimate the orientation.

The tool *CRISPR-evOr* is provided within the *SpacerPlacer* framework at `https://github.com/fbaumdicker/SpacerPlacer`. We provide orientation predictions for all groups and all tools, accession numbers and some useful information in a csv table in the supplementary material.

The input into the algorithm is a group of spacer arrays. We show examples for such groups in the github repository and in Figure 1, 2 and 3a) (already aligned and relabeled). *SpacerPlacer* supports the input of array groups as fasta of the array DNA sequences (of the spacers, repeats are removed) and groups of arrays with already fixed spacer ids. The spacer DNA sequences can be extracted from CRISPRCasdb and CRISPRCasFinder or CRISPRidentify output. Some scripts for the conversion and pre-processing (such as clustering based on spacer overlap) for CRISPRCasFinder output are provided as options of *SpacerPlacer* and available in the github repository.

For a reliable acquisition-orientation prediction, some amount of overlapping spacers and spacer diversity are required.

Description of orientation algorithm. Before starting the reconstruction process we identify and relabels unique spacers within the group with integers, as is done by *SpacerPlacer* [13]. If no tree was provided, the reconstruction is preceded by the estimation of a suitable phylogenetic tree based on the CRISPR arrays as described in [13].

Afterwards, *CRISPR-evOr* follows a three step strategy.

First, we reconstruct the ancestral states of the provided spacer data, in the orientation it is provided in, with *SpacerPlacer*. The computed maximum likelihood L_{forward} is saved for later use.

In a second step, we *reverse* the spacer integer sequences (not the DNA sequence) of each sample. This is equivalent to

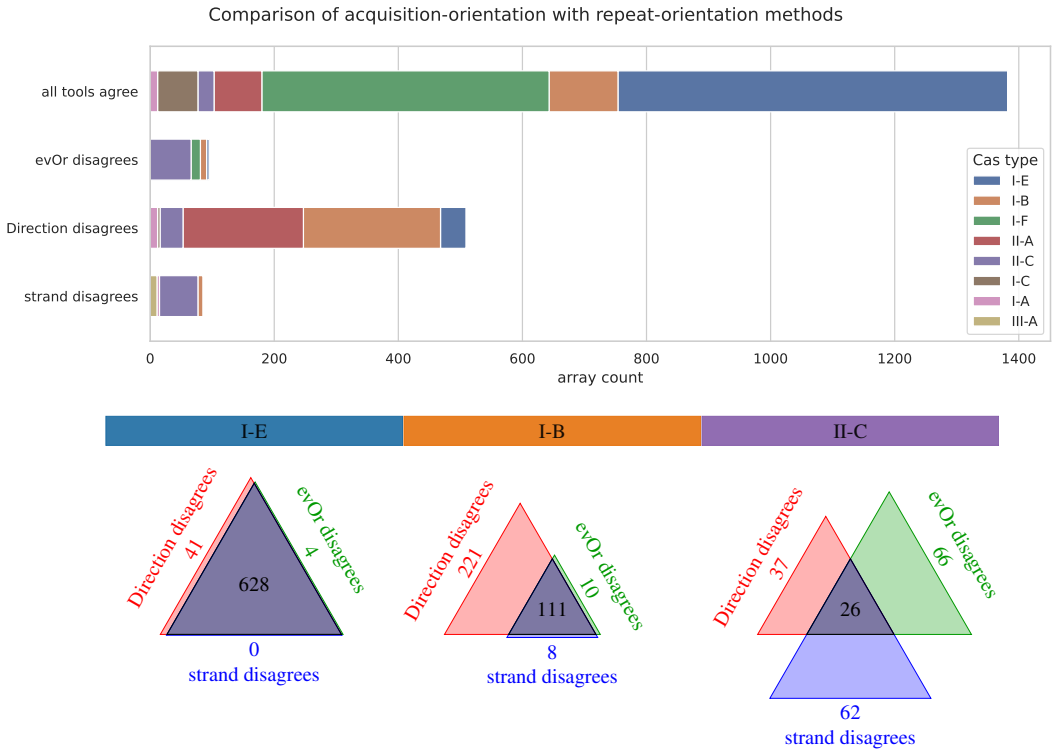


Fig. 4. Comparison with repeat based orientation methods. We show the agreement of orientation predictions between CRISPRDirection, CRISPRstrand and *CRISPR-evOr* where all methods have high confidence broken down according to Cas type. Cas types were filtered as in Figure 3b). For a few subtypes in the dataset, no (or only a few) high confidence predictions are available. At the bottom, we show Venn diagrams of the same data for three interesting subtypes with distinct behavior. Clearly, all methods agree quite often, especially for well-researched Cas types like I-E and I-F. However, there are substantial differences for predictions for type I-B, II-A and II-C. This indicates differences in the characteristics used for orientation prediction such as their repeats, leader and acquisition behavior. Moreover, since CRISPRDirection and CRISPRstrand were developed/trained using the more prevalent and well-studied Cas types, they may not be well-suited for the less common and less researched Cas types. Venn diagramms for the remaining types can be found in Supplementary Fig. S5.

reversing the orientation of *all* arrays in the dataset. We then estimate the ancestral reconstruction with *SpacerPlacer* in exactly the same way as in the first step. Again, we save the computed maximum likelihood L_{reverse}. Note, that if the tree was estimated based on the pairwise distance approach from *SpacerPlacer*, the tree estimation has to be repeated as well, since the distances are heavily dependent on spacer order.

In the third step, we compare the maximum likelihoods computed for each direction by taking the ratio of both likelihoods. If the ratio exceeds, or falls below, an empirically chosen threshold, *CRISPR-evOr* predicts either "forward" (input, step 1) orientation or "reverse" (reversed input, step 2) orientation respectively. If the logarithm of the ratio is too close to 0 according to the threshold, no confident prediction can be made and *CRISPR-evOr* returns "nd" for "orientation not determined".

Likelihood comparison and measuring confidence. The *SpacerPlacer* framework allows us to obtain a forward maximum likelihood L_{forward} and a reverse maximum likelihood L_{reverse} in steps 1 and 2. Since we use the same parameters for the reconstruction and either use a provided tree (which is independent of the spacer sequences), or, if no tree is provided, estimate the trees for each direction individually, we can compare both likelihoods in a meaningful likelihood ratio

$$L_{\text{ratio}} := \log L_{\text{forward}} - \log L_{\text{reverse}}.$$

To quantify the certainty of *CRISPR-evOr* we introduce a confidence threshold $c \geq 0$. However, as the likelihood is non-canonical the asymptotic distribution of the likelihood ratio is unknown. Therefore, we rely on empirical results in simulations and on available real data (see next paragraph) to determine a suitable confidence threshold c to make reliable decisions.

Computing the L_{ratio} results in one of two cases:

1. If $|L_{\text{ratio}}| > c$, one of the reconstructions can be confidently determined as the more likely according to the parametric model. Then, the orientation is determined by the sign of L_{ratio}.

2. If $|L_{\text{ratio}}| \leq c$, the likelihoods of the reconstructions are too close together and no confident prediction can be made. *CRISPR-evOr* returns "not determined".

A particularly common subgroup of uncertain orientation predictions is $|L_{\text{ratio}}| = 0$. Two such examples are shown in Supplementary Fig. S2. In this case, the reconstructed events with forward orientation are exactly as likely as the reconstructed events with reverse orientation according

to the parametric model. Note, that *SpacerPlacer* makes no difference between individual spacers (or arrays) but investigates only the retention of spacers and the number and location of deletion events [13]. Thus, perfectly symmetric arrays (not considering spacer labels) will result in the same likelihoods and return $L_{\text{ratio}} = 0$. If the first and last spacer in each sample are equal this is obviously the case. However, symmetry also occurs for arrays where the events (not considering spacer labels) before/after the first/last equal spacer are identical for both reconstructions. Naturally, besides symmetry in the spacer arrays, this depends heavily on the underlying tree. However, for such datasets *CRISPR-evOr* might be able to provide a prediction if the set of spacer arrays can be extended into a more diverse/less symmetric group.

Simulation-based benchmarking. We assess the performance of *CRISPR-evOr* in a suitable setting of three sets of simulated data (see methods) as in [13]. Note, that since every discussed orientation tool relies on distinct data types, it is not feasible to compare and test the performance of all discussed tools in a fair simulation setting.

In simulations along coalescent trees and moderate spacer acquisition and deletion parameters (dataset A) we found that *CRISPR-evOr* can be completely relied upon with 100% of the decision being correct for decision threshold $c = 0$. Furthermore, almost all simulations exceed any reasonable choice of threshold c. To investigate the reliability of *CRISPR-evOr* in a more realistic setting (dataset B), we evaluated *CRISPR-evOr* in two ways. We tested *CRISPR-evOr* providing the original trees (inferred from core genes in [13] and upon which we simulated the dataset). However, since reliable trees are not always available, we also tested the performance based on trees inferred by *SpacerPlacer* based on the spacer arrays themselves. In both of these more challenging settings *CRISPR-evOr* proved to be very reliable. When using the original tree and decision threshold $c = 0$, we found that 97.8% of the predictions were correct. Of the remaining 2.2%, only 0.2% give the wrong orientation prediction and 2% likelihood ratio exactly 0, i.e. no or symmetric events. When using the trees estimated based on the spacer arrays, we found that 95.5% of the predictions were correct and 1.5% have likelihood ratio exactly 0, similar to before. As the estimated trees based on the arrays can be of low resolution and diverge from the true tree, especially for groups with few observed evolutionary events, it comes at no surprise that *CRISPR-evOr* predicted the wrong orientation for 3.0% of the groups. However, imprecise tree inference does not change the underlying observed events and thus does not change results by a huge margin.

Naturally, when a decision threshold $c > 0$ is applied, the percentage of correct and wrong predictions decreases and some of the orientation predictions are labeled as uncertain instead. We report the results on the above simulation datasets with a conservative choice of $c = 5$ in the Supplementary Note 1B.

Evaluation of acquisition-orientation on real data. In Fehrenbach et al.'s CRISPRCasdb dataset, we found that many groups, e.g. the group shown in Figure 1, have high $|L_{\text{ratio}}|$ enabling *CRISPR-evOr* to make a clear decision for one orientation, when comparing both reconstructions. However, for array groups where limited or erratic evolution results in lower likelihood ratios decisions can be more difficult and are not always obvious (Figure 3a)). Thus, we set a conservative default choice of $c = 5$ by manually investigating the CRISPRCasdb data decisions with lower $|L_{\text{ratio}}|$.

We evaluate the performance of *CRISPR-evOr* by comparing our predictions to PAM-orientation prediction by Vink et al. and the leader- and repeat-orientation methods CRISPRDirection [19] and CRISPRstrand [17] which are part of CRISPRCasFinder [18] and CRISPRidentify [20], respectively. The evaluation of PAM-orientation versus *CRISPR-evOr* and CRISPRDirection and CRISPRstrand versus *CRISPR-evOr* are performed on different datasets as reported in methods and in the respective figure captions.

Note that *CRISPR-evOr* considers groups of arrays, whereas the PAM-orientation and leader- and repeat-orientation methods consider single arrays. In all figures here, we show the orientation predictions for individual arrays. We show the results in terms of groups in Supplementary Fig. S3 and S4. For additional remarks about how we arrive at group orientation predictions for array based methods, see Supplementary Note 1C. Moreover, we show pairwise comparisons between all tools in Supplementary Fig. S6 - S13.

PAM- & acquisition-orientation are confident and agree for large parts of the dataset. The number of arrays with confident predictions far exceeds those with uncertain predictions. However, both tools have a significant number of arrays (36.5%) where at least one of the tools is not confident in its predictions (Figure 3b)). As can be seen, PAM-orientation is confident on more arrays (89.3%) compared to *CRISPR-evOr* (68.6%). This is primarily due to many array groups that exhibit minimal or no evolution (113 groups with 471 arrays, 68.1% of all uncertain array predictions by *CRISPR-evOr*), making it challenging to predict the acquisition-orientation.

Additionally, we show in Figure 3b) that *CRISPR-evOr* and PAM-orientation are almost always in agreement for abundant types I-E, I-B, I-F and II-A. With the only possible exception of type II-C, where PAM-orientation and *CRISPR-evOr* disagree in 21.3% of cases. However, the number of available groups and arrays of type II-C (178 arrays) is quite small. *CRISPR-evOr* is not confident in available type III-B groups as only one of them shows *any* evolution.

Acquisition-orientation provides confident and reliable predictions compared to repeat based methods. When we applied *CRISPR-evOr* on the CRISPRCasdb dataset, we found that our method is confident for 63.7% of the arrays (55.4% of the groups), whereas CRISPRDirection and CRISPRstrand are confident on 65.3% and 83.5% of the arrays, respectively. While, *CRISPR-evOr*, CRISPRDirection and CRISPRstrand are confident on the same samples for 35.4% of the arrays (Supplementary Fig. S6, S7), there is a substantial amount of samples (28.3%) where either

CRISPRDirection and/or CRISPRstrand are uncertain but *CRISPR-evOr* is confident. Thus, *CRISPR-evOr* allows for confident orientation predictions for many arrays, where other methods are unreliable.

Among the groups where predictions of *CRISPR-evOr* are not confident (231 of 518 groups with 2152 of 5934 arrays), the number of groups/arrays with $L_{\text{ratio}} = 0$ far exceeds the number of groups/arrays with some amount of evolution but no confident prediction (48 groups, 671 arrays), i.e. $0 < |L_{\text{ratio}}| \leq c$. Across the CRISPRCasdb dataset, 35.3% (183) of the groups, consisting of 25.0% (1481) of the arrays have a likelihood ratio of 0 as they carry little to no diversity in spacer content or exhibit strong symmetry in forward and reverse reconstructions. We believe that besides inactive CRISPR systems, global sampling biases towards more closely related strains contribute to this lack of diversity among spacer arrays. However, as our evolutionary approach considers the information of all arrays, many of the array groups that currently result in a zero or low likelihood ratio can enable confident prediction by *CRISPR-evOr* in the future when additional arrays become available. Currently, on the subset of groups with sufficient non-symmetric diversity to use *CRISPR-evOr*, i.e. all 4453 arrays (335 groups) with $|L_{\text{ratio}}| > 0$, *CRISPR-evOr* is able to predict the orientation of 85.0% (3782) of the arrays (85.7% (287) of the groups) confidently.

The repeat-orientation tools provide an estimate based on a single array and not a group of arrays. However, within our groups CRISPRstrand's predictions are always either all uncertain or all confident and consistent for closely related arrays with the same consensus repeat. In contrast, while CRISPRDirection's predictions are consistent for all but 3 groups, sometimes only a few arrays per group provide a confident repeat-based orientation prediction. Thus, in most cases, the predictions of single or few arrays could be extended to the whole group. In Supplementary Fig. S14 we show a jitterplot of the fraction of confident predictions for each group for all three array-based tools.

To compare the contradicting predictions of the different tools, we performed a threefold comparison between *CRISPR-evOr* and CRISPRDirection and CRISPRstrand on the subset of our CRISPRCasdb dataset where *all* three tools have confident, but not necessarily identical, orientation predictions (Figure 4, Supplementary Fig. S5). This subset corresponds to the upper green column in Supplementary Fig. S6, S7, excluding rare types, amounting to 2071 arrays.

We find that for common well-studied Cas types like I-E and I-F, all tools are typically in agreement. For other systems, especially for type II-A and I-B, CRISPRstrand's and CRISPRDirection's prediction are more frequently in conflict than in agreement. This is remarkable since CRISPRDirection and CRISPRstrand rely mostly on similar information, i.e. the repeat mutation structure, although CRISPRDirection uses leader-based criteria as well. The independent evolutionary approach of *CRISPR-evOr* is able to resolve these conflicts. In general, our predictions are more aligned with CRISPRstrand's predictions than CRISPRDirection's predictions (Figure 4, Supplementary Fig. S5).

The picture is different for Cas type II-C, where CRISPRDirection, and CRISPRstrand often disagree with each other, but *CRISPR-evOr* does not consistently align with either tool. Furthermore, *CRISPR-evOr* disagrees in many cases where CRISPRstrand and CRISPRDirection are in agreement. While this pattern (see Figure 3b)) might suggest differences in the acquisition process or the repeats specific to type II-C arrays, it could also indicate that the repeat-based methods are not well-optimized for this CRISPR type.

Discussion

Here we demonstrate that the polarized insertion of spacers enables inference of the orientation of CRISPR arrays using evolutionary likelihood calculations. Compared to sequence-based methods, this is a completely independent approach, which not only allows to predict the orientation of previously unpredictable arrays with high confidence, but also to reject or confirm previous sequence-based CRISPR array orientation predictions.

Challenges in accurately predicting the array orientation

An advantage of our evolutionary approach in comparision with other approaches is that the detection of leader, repeat, and PAM sequences, which can be unreliable due to their inherent variability, does not influence our predictions. For example, some CRISPR-Cas systems do not carry a leader [9]. Orientation decisions based on repeats are often reliable, but also often lack confidence, particularly for short CRISPR arrays with few repeats [18, 20]. Similarly the transcription orientation of the Cas genes and the array orientation frequently but not necessarily align. Moreover, not for all CRISPR arrays Cas genes can be found nearby, and if so, CRISPR-Cas systems (e.g. I-A, II-C2) can also contain Cas genes that are transcribed in different directions [4].

For PAM-based orientation predictions, efficiently and reliably identifying protospacers and PAMs remains a challenge and depends on the availability of appropriate metagenomic datasets. Nonetheless, in those cases where this is possible we consider the orientation inferred from PAMs to be a reliable technique for determining the array direction, particularly when the results are based on a multitude of spacers within an array. However, one has to consider that the location and properties of PAM sequences are subtype specific. For example, the PAM is known to be on the 5' end for type I, type IV and V, and on the 3' end for type II systems where the PAM is also strain- and not repeat-specific, and not all types have (known) PAMs [11, 28, 29].

An evolutionary approach for array orientation prediction

The acquisition-orientation considered here is a distinct concept independent of (but correlated with) the leader- and repeat-orientation concepts.

Unlike all other tools discussed, *CRISPR-evOr* requires multiple CRISPR arrays with some amount of overlap and differences, i.e. active evolution, and naturally can not provide an orientation estimate for a single array or a set of identical

arrays. We thus recommend to identify related strains and include their CRISPR arrays to the analysis to generate the most reliable prediction. In CRISPRCasdb, we found a substantial amount (see Figure 3b) and Supplementary Fig. S6, S7) of closely related arrays which do not show observable evolution. This might be due to sampling biases in the database towards almost identical strains or due to CRISPR systems that evolve slowly or lost their acquisition machinery. Our approach to predict orientation based on the diversity of CRISPR arrays is not limited to the concrete likelihood calculation based on the evolutionary block deletion model in *SpacerPlacer*. In principle, any evolutionary model can be used. For example, the most parsimony approach implemented in the CRISPR Comparison Toolkit (CCTK) [30] could be used to reconstruct ancestral arrays for both orientations and identify the more parsimonious direction.

Variations of the acquisition and deletion mechanisms between different types, which differ from model assumptions could impact any evolutionary approach. While the deletion mechanisms are rather universal across different subtypes [31], some Cas subtypes, do not always strictly follow polarized insertions. However, polarized insertions seems to be the predominant mode of spacer acquisition [31], although in some studies ectopic insertions within the array have been observed for I-A [32, 33] and II-A [34–37].

Indeed, *CRISPR-evOr* is more decisive on simulated data, based on a simple evolutionary model, than real data, suggesting that CRISPR arrays are subject to more complex evolutionary processes. This not only includes uncommon events like ectopic insertions, duplicate events, and array collapse, but also sequencing errors, selection pressures, and changes in the environment which can all lead to unexpected patterns. However, spacer duplications and other complex evolutionary events are classified as such by *SpacerPlacer* and thus do not directly impair the orientation prediction of *CRISPR-evOr*. The strong performance in simulations in combination with the agreement with sequence based prediction methods for most CRISPR system types underscores the power of our evolutionary approach.

The three main requirements for a reliable orientation prediction of our method are (i) polarized acquisition of new spacers, (ii) active evolution of the CRISPR array, and (iii) a sufficiently diverse set of different sequenced CRISPR arrays. Ongoing sequencing and sampling efforts will extend the available datasets and generate larger more diverse groups of related arrays, and, in turn, allow *CRISPR-evOr* to make even more confident and reliable predictions in the future. However, *CRISPR-evOr* is already able to make confident decisions based on groups with only three arrays (with sufficient diversity).

Comparison of orientation predictions across Cas types

We find that the orientation predictions of the abundant Cas types I-E and I-F show a strong consensus among all investigated orientation concepts. The consensus is to be expected as these abundant types are well-researched and served as blueprint for typical CRISPR behavior in terms of evolution, expression, transcription and repeat and leader characterization. CRISPRDirection, CRISPRstrand and CRISPRleader rely on leader and repeat attributes that were defined based on these most abundant types, which are also a substantial part of the training and test datasets for CRISPRstrand and CRISPRdirection. Similarly, *CRISPR-evOr* is reliant on polarized insertion, which is a behavior that characterizes (almost) all types of CRISPR arrays but was identified and experimentally confirmed particularly for type I.

Interestingly, for types I-B and II-A, *CRISPR-evOr*, PAM-orientation and CRISPRstrand agree in their prediction for almost all arrays, while CRISPRDirection disagrees with them. Thus, for those types, CRISPRstrand is likely more reliable than CRISPRDirection. Since the orientation predictions are reinforced by three different concepts based on the PAM, the repeats and the evolutionary likelihood, we conclude that CRISPRDirection is likely misclassifying the corresponding arrays. This is in line with the observation that CRISPRDirection is also confident for fewer arrays than CRISPRstrand (Supplementary Fig. S6, S7). While wrongly predicted orientations by CRISPRDirection and consequently in CRISPRCasdb have already been noted for some strains and systems [17, 38], our results indicate that - at least among the considered dataset of arrays, with confident predictions by CRISPRDirection, CRISPRstrand and *CRISPR-evOr* - the orientations of 63.1% of type I-B and 71.6% of II-A arrays on CRISPRCasdb are incorrect. Correctly inferred orientations are crucial for a multitude of subsequent analyses, e.g. regarding array transcription, or protospacer identification [39–42]. Thus, for the afore mentioned and related CRISPR types we advise reassessing the corresponding data and experiments to verify if the conclusions remain valid when the orientation of many arrays has to be reversed.

Among the three tools, the greatest divergence in predicting the array orientation arises with type II-C (Figure 3b) and Figure 4, Supplementary Fig. S5). In one study, II-C arrays have been reported to favor acquisition at the downstream end of the array (relative to transcription direction) [43]. Those arrays also lacked recognizable leader sequences. Moreover, Type II-C has been shown to not only have leader promoters, but promoters in the repeats [43, 44], which lowers the selective pressure to insert new spacers at the leader end, but might also cause deviations from patterns observed in leader and repeats of other systems. *CRISPR-evOr* performs well on available II-A arrays, which supports results showing that type II-A acquisition is polarized [34]. However, ectopic insertions have been reported for type II-A [34–36, 45], although they occur less frequently than polarized insertions and only when specific regions of the leader are mutated or lost [32–34].

Moreover, a manual inspection of the reconstructions of the groups of II-C arrays with confident predictions showed normal (polarized) evolutionary behavior and thus sensible acquisition-orientation predictions. Although a third of all available arrays of type II-C in the dataset showed no evolution at all and allowed no confident predictions by *CRISPR-evOr*, this does not indicate that CRISPR arrays of type II-C systems

might evolve more slowly, as other CRISPR types have similar fractions of arrays without diversity.

Conclusions and future perspectives With *CRISPR-evOr* we provide a reliable tool to predict the acquisition-orientation of CRISPR arrays. Our results suggest to treat the orientations of CRISPR arrays from CRISPRCasdb, at least for some systems, with caution.

Among the different orientation concepts, acquisition-orientation is most directly related to the adaptive evolutionary dynamics of spacer arrays and thus carries an additional ecological importance. The acquisition-orientation is crucial to understand the interaction between the environment and the CRISPR-Cas system. This includes especially the acquisition and deletion mechanisms, and their defensive and/or regulatory function in bacteria and archaea. In particular, to assess the relevance and protective capabilities of specific spacers and the ecological importance of their targets, it is essential to know whether spacers have been acquired recently or in the more distant past [46].

As the availability of CRISPR arrays continues to grow, especially with the increasing number of long-read sequencing projects, *CRISPR-evOr* will allow to investigate CRISPR arrays for various CRISPR-Cas types in the future, including type III CRISPR arrays that have been found to be distinct in their spacer acquisition [35, 41].

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