Large-scale motif discovery using DNA Gray code and equiprobable oligomers

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ABSTRACT

Motivation: How to find motifs from genome-scale functional sequences, such as all the promoters in a genome, is a challenging problem. Word-based methods count the occurrences of oligomers to detect excessively represented ones. This approach is known to be fast and accurate compared with other methods. However, two problems have hampered the application of such methods to large-scale data. One is the computational cost necessary for clustering similar oligomers, and the other is the bias in the frequency of fixed-length oligomers, which complicates the detection of significant words.

Results: We introduce a method that uses a DNA Gray code and equiprobable oligomers, which solve the clustering problem and the oligomer bias, respectively. Our method can analyze 18 000 sequences of ∼1 kbp long in 30 s. We also show that the accuracy of our method is superior to that of a leading method, especially for long-sequence data and small fractions of motif-containing sequences.

Availability: The online and stand-alone versions of the application, named Hegma, are available at our website: http://www.genome.ist.i.kyoto-u.ac.jp/~ichinose/hegma/

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1 INTRODUCTION

The technological development of next-generation sequencing has enabled us to obtain genome-scale promoter sequences (Wakaguri et al., 2008). The first step toward unraveling the regulatory mechanisms from such large-scale data is to identify cis-regulatory motifs. Existing computational algorithms used for motif finding may be categorized into three classes: (1) motif discovery from promoter sequences in a single genome (Sandve and Drabblis, 2006); (2) phylogenetic footprinting that uses promoter sequences from multiple species (Das and Dai, 2007); and (3) motif search relying on known motif models, such as all the promoters in a genome, is a challenging problem. First, it is not trivial to cluster similar oligomers into fewer groups. Fundamentally, a word-based method initially detects interesting oligomers without allowing any substitutions, whereas a motif is typically a set of similar oligomers that contain some variations among them. Hence, we need to apply a clustering method to gather similar oligomers. However, the computational cost rapidly increases with the number of initial oligomers or the degree of allowed variations. Second, the detection of significantly abundant oligomers is complicated by the variable background frequencies of different oligomers with a fixed length. For example, the background frequencies of AT-rich and GC-rich oligomers can differ extensively in human promoter sequences. Moreover, the difference becomes more remarkable for longer oligomers. Thus, we have to carefully evaluate the statistical significance of over-representation of particular oligomers in large-scale data.

Here, we report a new motif discovery method that can analyze tens of thousands of DNA sequences each ∼1 kbp long. We solve the first problem by using a DNA Gray code [originally proposed by Gray (1947), see also Er (1984)]. The DNA Gray code is an ordering of oligomers in which adjacent oligomers differ from each other by only one nucleotide. Since neighboring oligomers in the DNA Gray code are similar to one another, we can solve the first problem by searching only neighborhoods within the DNA Gray code. To solve the second problem, we use ‘equiprobable’ oligomers, the lengths of which are variably adjusted so that every oligomer should have an approximately equal background probability. It is easily shown that the equiprobable oligomers can be naturally combined with the DNA Gray code.

We implement our motif discovery method in C to produce the computer program named ‘Hegma’ and evaluate the performance of Hegma by using a known database, cisRED (Robertson et al., 2006). The benchmark test indicates that in most situations Hegma
outperforms Weeder (Pavesi et al., 2004), the best existing word-based motif discovery tool (Tompa et al., 2005). As Hegma is three to four orders of magnitude faster than Weeder, Hegma may be applicable to unprecedented scales of data analyses.

2 METHODS

2.1 DNA Gray code

A Gray code is a coding system of binary numbers in which adjacent numbers differ by only one bit. Although Gray has initially proposed this code as such binary numbers (Gray, 1947), we can easily extend it to quaternary numbers (Er, 1984) to be applied to a DNA sequence. The DNA Gray code can be constructed iteratively from monomers to arbitrary length oligomers. Consider a monomer code (A,G,C,T). This code is obviously a Gray code because adjacent monomers differ by one nucleotide. Note that we regard the last monomer to be adjacent to the first monomer, and this circularity holds for longer oligomers. We prepare four copies of the monomer Gray code and concatenate them with each nucleotide, but in the case of the last row, the copies are arranged in the reverse order. This procedure yields the dimer Gray code as illustrated in Figure 1. In the same manner as the dimers, we can construct the DNA Gray code of k-mers (k > 1) by preparing four copies of the (k − 1)-mer Gray code, two of which are reversed and concatenating them to each nucleotide.

In general, if the (k − 1)-mer code is a Gray code, the k-mer code constructed by the above procedure is also a Gray code. This fact can be understood from the following observations. We can partition the k-mer Gray code into four regions in which the first nucleotides in each region are identical. Inside each region, the oligomers are arranged in Gray code order because the first nucleotides are identical and the others are the (k − 1)-mer Gray code. On the other hand, two oligomers at both sides of a boundary between neighboring regions are identical except for the first nucleotides because of the reverse copy. Consequently, the k-mer code is inductively a Gray code as the monomer code is a Gray code. The DNA Gray code has an ordered tree structure as a consequence of the construction process mentioned above (Er, 1984). This implies that we can apply the depth-first search algorithm to the tree to naturally order oligomers of variable lengths. This feature is important in combining the DNA Gray code with the equiprobable oligomers, as discussed later in Section 2.3.

The Hamming distance between oligomers located at a distance d in the DNA Gray code is smaller than or equal to d. In this regard, when we extract some consecutive oligomers from the DNA Gray code, those oligomers are similar to one another. However, all similar oligomers are not necessarily in a neighborhood in the DNA Gray code, i.e., two oligomers having a small Hamming distance can be located at distant positions. Nevertheless, we can show that the property of the neighboring similarity is beneficial for efficient data processing compared with conventional methods (Section 3).

![Fig. 1](https://academic.oup.com/bioinformatics/article-abstract/28/1/25/219964/487x275)

**Fig. 1.** Construction process of the DNA Gray code of dimers. The ordinary and reverse copies in the second row are copied from the monomer Gray code in the first row. The concatenation of the first and second rows yields the dimer Gray code shown in the third row.

2.2 Shift detection

Two oligomers with a shift relation, for example ACGGT and CGGTC, are similar to each other in the sense of edit distance, although the Hamming distance between them is large. Because of the large Hamming distance, we cannot immediately detect the similarity between such oligomers in the DNA Gray code. Fortunately, however, we can detect the shift relations of the oligomers at a low cost by taking advantage of the feature that the DNA Gray code is left shift continuous.

Let S be a semi-infinite sequence, S = s0s1s2... ∈ {A,G,C,T}. The left shift σ of the sequence is defined by

\[\sigma(S) = s_1s_2s_3... = s_{k+1}s_{k+2}...\] (1)

Note that the left shift is the inverse of the construction of the DNA Gray code; in the construction process, we concatenate oligomers with each nucleotide, whereas we remove the first nucleotides from the oligomers in the left shift.

To explain the left shift continuity, we introduce a real-valued representation of the sequence in the DNA Gray code. Let G = \{(x0, x1, x2,..., xk−1) \} be a DNA Gray code with N = 4^k oligomers, where gi is an oligomer of length k, \(gi = x_0x_1x_2...x_{k−1}\). The real-valued representation \(ϕ_k\) of \(gi\) is defined by:

\[ϕ_k(g_i) = \sum_{j=0}^{k−1} x_j 2^j \in [0, 1].\] (2)

In general, there is also a real-valued representation \(ϕ\) of a semi-infinite sequence \(S = ϕ(S)\) as \(k \to ∞\). Our aim here is to show the function \(ϕ\) that corresponds to the left shift \(σ\) in the real-valued domain.

In order to understand the left shift function \(ϕ\), we consider the construction process of the DNA Gray code in the real-valued domain, as shown in Figure 2. The copies and reverse copies in the construction process correspond to the linear maps that have positive and negative slopes in the real-valued domain, respectively. Therefore, the process is expressed as shown in Figure 2a. Since the left shift is the inverse process of the construction, we can obtain the left shift function as the inverse map, as shown in Figure 2b. This function is equivalent to the composition map of the tent map well known in chaos theory (Alligood et al., 1997).

It should be noted that the function \(ϕ\) is continuous. The left shift continuity implies that the image mapped from a contiguous region in the DNA Gray code, which corresponds to a set of similar oligomers, is also contiguous. If the functions were discontinuous, a contiguous region would be mapped to scattered regions. The left shift continuity ensures that we can obtain a single region whenever a contiguous region is mapped.

Figure 3 illustrates two examples of contiguous regions \(r_1\) and \(r_2\), and their images \(r_1'\) and \(r_2'\). The region \(r_2\) overlaps with the image \(r_1'\) (Fig. 3b), which corresponds to the left shifts of \(r_1\) (Fig. 3a). Since this implies that \(r_2\) is included in the left shifts of \(r_1\), we can judge that those regions have a shift relation. Thanks to the left shift continuity, a shift relation can be detected by mapping only two oligomers at the beginning and end of the region even though the contiguous region is composed of many oligomers.

To detect overlapped pairs in a set of contiguous regions, we compare the regions with a sorted list of their images. We can compare those lists in...
A and C with the 0-th order Markov model as the background probability. The background probability is a model that represents an intrinsic property $I_S$ of an oligomer $S$ but whose background probabilities are adjusted to be nearly identical to one another. This problem, we propose equiprobable oligomers whose lengths are variable, whose background probabilities causes statistical bias in the significance detection. To overcome this problem, we use the 0-th order Markov model with $I(S) = -\log_2 P_S$ and $P(S)$ is the background probability. Let $S'$ be the oligomer in which the right-most nucleotide is removed from $S$. We define the equiprobable oligomer $S$ such that it has the following property.

$$I(S') < \theta$$  \hspace{1cm}  (3)

where $\theta$ is a threshold parameter.

As an example, we consider equiprobable oligomers that consist of only A and C with the 0-th order Markov model as the background probability. In the 0-th order Markov model, the background information content $I(S)$ of an oligomer $S$ is expressed as the sum of the background information contents of individual nucleotides, i.e., $I(S) = \sum_{i=1}^{n} I(G_i)$. Figure 4 illustrates such equiprobable oligomers. Each box corresponds to a nucleotide and its height is drawn to be proportional to the information content of that nucleotide. Therefore, when the (downwardly) headed boxes exceed the threshold $\theta$, the column of those nucleotides becomes an equiprobable oligomer. All the equiprobable oligomers do not have exactly the same probability; for example, $I(\text{AAA})=8$ and $I(\text{CCC})=9$. However, the equiprobability is considerably improved compared with fixed-length oligomers, especially in the cases of longer oligomers and a higher order Markov model. The validity of the digitizing approximation is discussed in Section S.1 in Supplementary Material.

Consider two oligomers, $S_1$ and $S_2$, such that $S_1$ is shorter than $S_2$. If $S_2$ is an equiprobable oligomer and $S_1$ matches a prefix of $S_2$, $S_1$ cannot be an equiprobable oligomer because $I(S_1)$ should be smaller than $\theta$ under the property of Equation (3). This observation implies that the set of equiprobable oligomers is a prefix code in which no oligomer matches a prefix of any other oligomer. Recall the feature that the DNA Gray code has the ordered tree structure. In the prefix code, a code word is always located at a leaf of the tree. Therefore, the equiprobable oligomers can be ordered on the tree and hence we can naturally combine the equiprobable oligomers with the DNA Gray code so that adjacent oligomers differ from each other by just one nucleotide up to the length of the shorter oligomer.

Algorithm 1 Display equiprobable oligomers with DNA Gray code

<table>
<thead>
<tr>
<th>procedure equigraycode(string $S$, boolean $f$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>if $I(S) \geq \theta$ then</td>
</tr>
<tr>
<td>print $S$</td>
</tr>
<tr>
<td>else</td>
</tr>
<tr>
<td>if $f$ then</td>
</tr>
<tr>
<td>equigraycode($S$ + “N”, true)</td>
</tr>
<tr>
<td>equigraycode($S$ + “G”, false)</td>
</tr>
<tr>
<td>equigraycode($S$ + “C”, true)</td>
</tr>
<tr>
<td>equigraycode($S$ + “T”, false)</td>
</tr>
<tr>
<td>else</td>
</tr>
<tr>
<td>equigraycode($S$ + “T”, true)</td>
</tr>
<tr>
<td>equigraycode($S$ + “C”, false)</td>
</tr>
<tr>
<td>equigraycode($S$ + “G”, true)</td>
</tr>
<tr>
<td>equigraycode($S$ + “N”, false)</td>
</tr>
<tr>
<td>end if</td>
</tr>
</tbody>
</table>

Algorithm 1 shows the recursive procedure that performs the depth-first search on the tree of the DNA Gray code. By calling $\text{equigraycode}(\text{\textquotedbl}true\text{\textquotedbl})$, one can display all of the equiprobable oligomers with the DNA Gray code. If we use the i.i.d. uniform distribution as the background model, we can obtain the DNA Gray code with a fixed length of $\theta/2$, because $I(S) = 2|S|$ in this case. Therefore, Algorithm 1 can generate the DNA Gray code as a special case.

2.4 Significance detection

We have now obtained the DNA Gray code of equiprobable oligomers. To detect significant motifs from a given set of sequences, we count the occurrences of equiprobable oligomers. Let $C$ be a set of occurrence counts of equiprobable oligomers:

$$C = \{c_0, c_1, \ldots, c_{|S|-1}\},$$  \hspace{1cm}  (4)

where $M$ is the number of equiprobable oligomers and $c_i$ is the count of the $i$-th oligomer in the DNA Gray code. We define a contiguous region $(c_i)$ as a cluster if it satisfies the following conditions.

$$c_i > 0, k \in (c_i).$$  \hspace{1cm}  (5)

The cluster is a set of similar oligomers that appear in the given sequences.

We detect the significance of the cluster by using its width $w = j - i + 1$ and the total count $o = \sum_{i \in (c)} c_i$. The null hypothesis is that the cluster is
obtained from random sequences generated by the background model. In the background model, the occurrence probability \( p \) of each oligomer can be approximated by \( p = 1/M \) because oligomers are equiprobable. Let \( q \) be the probability of an oligomer that occurs at least once. Thus, \( q \) is expressed as \( q = 1 - (1 - p)^T \), where \( T \) is the total number of oligomers in the given sequences. The random width \( W \) against \( w \) can be understood as Bernoulli trials where there are \( W \) successes with the probability \( q \) between two failures. Therefore, the probability distribution of \( W \) is a geometric distribution represented by:

\[
P(W) = q^W(1-q)^T.
\]

(6)

Since \( O \geq w \), the random total count \( O \) against \( w \) is conditioned by the width \( w \). If there is no constraint, the probability distribution of \( O \) is a binomial distribution with the success probability \( wp \) and the number of observations \( T \). The conditional probability distribution is represented by:

\[
P(O\mid w) = \text{Bin}(O\mid wp, O \geq w),
\]

(7)

where Bin is the binomial distribution:

\[
\text{Bin}(O) = \binom{T}{O} wp^O (1-wp)^{T-O}
\]

(8)

Using these distributions, we define the \( p \)-value \( pv \) of a cluster by:

\[
pv = P(W \geq w\mid O \geq w).
\]

(9)

Since there are many clusters in the set of occurrence counts \( O \), a large number of significance tests must be involved. To reduce the false discovery rate, we use the \( e \)-value \( ev \) instead of the \( p \)-value, which is adjusted by the number of equiprobable oligomers. \( M \) as follows.

\[
ev = P(W \geq w\mid O \geq w)M
\]

(10)

If \( ev \) is smaller than a significance level \( \alpha \), the null hypothesis is rejected and hence the corresponding cluster is judged to be significantly enriched.

### 2.5 Summary of methods

The flowchart shown in Figure 5 summarizes our motif discovery procedure. The parameter that characterizes each process is presented beneath the description of the process.

1. **Threshold parameter \( \theta \)**: the threshold parameter \( \theta \) is critical in our method because it regulates the probability of equiprobable oligomers \( p \). Empirically, we can obtain good results when we set \( p = 1/L \), where \( L \) is the total sum of the lengths of the input sequences. Therefore, in the application, \( \theta \) is automatically adjusted in accordance with the input sequences, such that \( \theta = \log(L) - \varepsilon \) (empirically, \( \varepsilon = 1 \)). The rationale behind this estimation is discussed in Section 5.2 in Supplementary Material.

2. **Order of Markov model \( m \)**: the background Markov model is constructed from the input sequences that include the motifs themselves. Since the regions occupied by the motifs are much smaller than the rest of the sequences, the background model can be properly estimated if \( m \) is small. The default value of \( m \) is fixed at 3.

3. **Significance level \( \alpha \)**: the significance level \( \alpha \) is not crucially influential in our method. We set the default value at 0.01 as a typical value.

4. **Number of shifts**: after finding significant clusters, we sort them in the ascending order of their \( e \)-values. We pick up each cluster in this order and look for other clusters that have a shift relation with it. The clusters thus found are merged into a single motif. This process is recursively performed. The depth of this recursion defines the number of shifts allowed. We set the default value for the depth at 3.

### 2.6 Data and statistics

As the benchmark data, we use the set of human promoter sequences in the cisRED database (Human v9.0, Robertson et al., 2006). The cisRED database consists of a set of promoter sequences and a set of motifs defined in the sequences, where each motif is conserved among several species and annotated according to the known motif database TRANSFAC (Wingender, 2004). The number of promoter sequences is 18,779. The total number of nucleotides is \(~47\text{Mb})\), of which valid (unmasked) nucleotides amount to \(~31\text{Mb})\). After removal of redundancy, the number of conserved motifs is 236,208 and the number of nucleotides occupied by the motifs is \(~2.3\text{Mb})\).

By comparing the sites predicted by our method with those listed in the cisRED database, we assess the performance of our method at two distinct levels, the nucleotide level and the site level. The statistics we use are essentially the same as those adopted by Tompa et al. et al. in their assessment strategy (Tompa et al., 2005). At the nucleotide level, each dataset consists of pairs \((i, p)\), where \( i \) is the sequence ID and \( p \) is the nucleotide position within the site. We denote the sets of known sites and predicted sites by \( \mathbf{K} \) and \( \mathbf{P} \), respectively. At the site level, each set consists of triples \((i, s, e)\), where \( i \) is the sequence ID and \( s \) and \( e \) are the start and end positions of the site, respectively. We denote the sets of known and predicted sites by \( \mathbf{K} \) and \( \mathbf{P} \), respectively.

At the nucleotide level, the true positive \( nTP \) is simply defined by:

\[
nTP = |\mathbf{K} \cap \mathbf{P}|
\]

(11)

where \( | \cdot | \) implies the size of the set. At the site level, the true positive \( sTP \) is expressed as:

\[
sTP = \max \{ u \in \mathbf{K} \mid (i, u, v) \in \mathbf{P}, u = v, o(s,v) \geq \text{len}(u)/4 \}
\]

(12)

where \( o(s,v) \) is \( \min(u,v) - \max(u,v) + 1 \) (overlap) and \( \text{len}(u) = u - v + 1 \) (length). This expression implies that \( sTP \) is the number of known sites that overlap with the predicted sites by at least one-quarter of the length of the known site.

The false positive and the false negative are defined as follows,

\[
sFP = |\mathbf{P} \setminus \mathbf{K} \cap \mathbf{P}|
\]

(13)

\[
nFN = |\mathbf{K} \setminus \mathbf{P}|
\]

(14)

where \( L \) is the number of valid nucleotides in the promoter sequences.

Of the above definitions, only the false positive at the site level \( sFP \) is different from that of Tompa et al. (2005). Tompa et al. allowed overlaps between the predicted sites and removed such sites from \( sFP \) if each site overlapped with a known site. In contrast, we use a slightly more stringent criterion to check whether the clustering of motifs is appropriately performed, i.e. we include the overlaps of the predicted sites in \( sFP \) even if the sites overlap with a known site.

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Fig. 5. Flowchart of the motif discovery. Each box that corresponds to a process presents the description (upper) and the parameter (lower) within it.
To examine the performance of our method, Hegma, we adopt the average site performance \( sASP \) at the site level, which is defined by:

\[
sASP = (sSn + sPPV)/2.
\]

In a similar way, we adopt the average site performance \( xASP \) at the site level, which is defined by:

\[
xASP = (xSn + xPPV)/2.
\]

3 RESULTS AND DISCUSSION

3.1 Performance evaluation with all motifs in cisRED

To examine the performance of our method, Hegma, we adopt the same evaluation scheme as that used by Tompa et al. (2005). To evaluate the effects of data size on the performance, we prepare sets of sequences that are randomly selected from the human promoter sequences of the cisRED database. In the following results shown in Figure 6, we prepare 10 sets for each number of sequences.

Figure 6a indicates that \( nPPV \) at the nucleotide level is insensitive to the variation in the number of sequences. In the default setting, our method adjusts the threshold parameter such that the equiprobable oligomers should have the probability \( p = 1/L \) under the background model, as discussed in Section 2.5. This adjustment maintains the null distribution at a constant precision, which accounts for the constant rate of false positive (or type I error) and hence nearly constant \( nPPV \). In contrast, \( nSn \) is improved as the number of sequences is increased. This improvement can be explained by the general characteristics of statistical analysis, where a larger data size leads to more precise results.

The results at the site level are similar to those at the nucleotide level except that \( xPPV \) decreases for larger numbers of sequences (Fig. 6b). This decrease in \( xPPV \) originates from overlaps between predicted sites, which augment \( xFP \) under our definition. Our method can detect a shift relation between overlapped sites and merge them. If this process were perfectly performed, the overlaps of the predicted sites would be repressed. However, we fail to eliminate all the overlaps partly because we restrict the size of shifts to 3 in the default setting. We impose this restriction to avoid the risk of merging unrelated motifs. Improved discrimination between related and unrelated motifs is one task to be explored in the future.

Figure 7 shows the memory usage and the calculation time. Calculations are made on a computer with 3 GHz Intel Xeon® with 16 GB memory running under Linux® 2.6. Both time and memory linearly increase with the number of sequences. It is noteworthy that we need only 30 s for calculation of the full data (18,779 sequences, 31 Mb). The memory usage of 1.1 GB is also sufficiently feasible for current conventional computers.

3.2 Performance evaluation with specific motifs

We compare the performance of our method to that of Weeder (version 1.4.2, Pavesi et al., 2004), a representative word-based method based on exhaustive enumeration with a limited number of mutations. We choose Weeder because it performed best in the assessment of Tompa et al. (2005).

Almost all the conventional tools, including Weeder, assume that given promoter sequences are derived from coregulated genes. This assumption implies that most of the given sequences have at least one specific motif that contributes to the specific regulation. Therefore, we prepare a set of sequences in which the fraction of sequences holding the motif is variably specified. We adopt the motif AhR as the specific motif, because it is the most frequent motif in the TRANSFAC annotations. Let \( R \) and \( U \) be the sets of sequences with and without the motif AhR, respectively. We select sequences from \( R \) and \( U \) according to a predefined percentage that we control. For example, when the total number of sequences is 1000 and the
The performance of Weeder under all other situations. The fractions of the motif-containing sequences are varied from 40% to 100%. The parameter setting of our method is default. See the text for the parameter setting of Weeder.

The results at the site level (Fig. 8b) are more remarkable than those at the nucleotide level. At this level, Hegma outperforms Weeder under all situations, including the case that 100% of the sequences contain the motif. The maximum of motif-containing sequences is 80%, we select 800 sequences from R and 200 sequences from U. In the following results, we fix the number of sequences at 1000. In order to evaluate the performance of single-motif detection, we regard only the known motifs as the right sites of the motif. An additional examination on a smaller ChIP-seq peak dataset also supports this conclusion as shown in Section S.3 in Supplementary Material.

We repeat the same analysis as mentioned above for the 10 most frequent motifs in cisRED (AhR, aMEF-2, POU2F1, Pax-5, DEAF-1, CREB, HNF-1a, DP-1, KRRC4 and POU3F2). Figure 9 summarizes the results for these 10 motifs at the nucleotide level by averaging their statistics. The detailed results for individual motifs together with the results of non-parametric statistical tests are presented in Section S.4 in Supplementary Material. Clearly, Hegma outperforms Weeder under all the situations tested. The results at the site level are also similar to those at the nucleotide level (data not shown). These observations imply that the performance of Hegma is more stable than that of Weeder regardless of the type of motif as well as the fraction of sequences that contain the motif. An additional examination on a smaller ChIP-seq peak dataset also supports this conclusion as shown in Section S.3 in Supplementary Material.

The average calculation time per dataset (1000 sequences) for Weeder is 10 h, whereas that for our method is only 1.4 s when tested under the same condition mentioned in Section 3.1 and averaged over 40 trials. Therefore, our method shows considerable advantage in calculation time as well.

3.3 Analysis of unannotated motifs

In Section 3.1, we regard the predicted sites that do not match any cisRED annotation as ‘false positives’. However, it is probable that some of them actually represent true motifs absent from the cisRED annotation. We then extract such unannotated motifs from all significant motifs predicted by Hegma in the full data of the cisRED promoters such that >95% of the sites comprising each motif do not overlap with any annotated sites. The number of all the predicted motifs is 7528 (composed of a total of 620,153 sites), of which the number of unannotated motifs is 1161 (36,443 sites). Figure 10 illustrates four examples of the unannotated motifs with the smallest $\alpha$-values in sequence logos (Schneider and Stephens, 1990).

The unannotated sites tend to be located in distal regions compared with all the predicted sites; the average position (±SD) of the unannotated sites is $-1140±894$ bp relative to the transcription start.
start sites, whereas that of all the predicted sites is $-737.5 \pm 837$ bp (p-value of t-test: $\approx 0$). The unannotated sites are a subset of the predicted sites and its complementary set is associated with the cisRED annotation. Therefore, this disparity suggests that the positions of the annotated sites in cisRED may have significant bias toward proximal regions. These observations may be interpreted as follows; it may be difficult for a phylogenetic footprinting approach, including cisRED, to detect conserved motifs in the distal regions, where the marked sequence divergence or the existence of repetitive elements hinders reliable sequence alignment compared with more conserved proximal regions (Suzuki et al., 2004). Therefore, our method can complement the phylogenetic footprinting approach to improve the overall sensitivity of motif discovery.

4 CONCLUSION

We have developed a large-scale motif discovery tool, Hegma, and shown that Hegma is not only applicable to large-scale data, but also can stably detect motifs even if only a small fraction of the examined sequences contain the motifs. Thus, Hegma is applicable to situations where the fraction of motif-containing sequences is uncontrollable, such as the detection of splicing enhancers or silencers in exon and intron sequences, or the detection of microRNA binding sites in UTR sequences. A huge number of such sequences have already been collected in databases. However, as our knowledge of those motifs is yet far from complete, it is difficult to know in advance the percentage of sequences holding the motifs. We consider that the speed and precision of Hegma would facilitate discovery of novel motifs from a heap of sequence data.

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