Estimation of Pairwise Sequence Similarity of Mammalian Enhancers with Word Neighbourhood Counts

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ABSTRACT

Motivation: The identity of cells and tissues is to a large degree governed by transcriptional regulation. A major part is accomplished by the combinatorial binding of transcription factors at regulatory sequences, such as enhancers. Even though binding of transcription factors is sequence-specific, estimating the sequence similarity of two functionally similar enhancers is very difficult. However, a similarity measure for regulatory sequences is crucial to detect and understand functional similarities between two enhancers and will facilitate large-scale analyses like clustering, prediction and classification of genome-wide data sets.

Results: We present the standardised alignment-free sequence similarity measure \(N2\), a flexible framework that is defined for word neighbourhoods. We explore the usefulness of adding reverse complement words as well as words including mismatches into the neighbourhood. On simulated enhancer sequences as well as functional enhancers in mouse development, \(N2\) is shown to outperform previous alignment-free measures. \(N2\) is flexible, faster than competing methods and less susceptible to single sequence noise and the occurrence of repetitive sequences. Experiments on the mouse enhancers reveal that enhancers active in different tissues can be separated by pairwise comparison using \(N2\).

Conclusion: \(N2\) represents an improvement over previous alignment-free similarity measures without compromising speed which makes it a good candidate for large-scale sequence comparison of regulatory sequences.

Availability: The software is part of the open source C++ library SeqAn (www.seqan.de) and a compiled version can be downloaded at: http://www.seqan.de/projects/alf.html

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1 INTRODUCTION

Mammalian organisms consist of several hundred different cell types. Every cell has the same repertoire of genes, however, only a subset will be expressed to enable cell-type-specific phenotypes. Many different factors regulate gene expression, of which genetically encoded transcriptional regulation seems to play the major part (Wilson et al., 2008). Sequence dependent gene regulation is mainly achieved through the binding of transcription factors at short DNA motifs. These transcription factor binding sites often occur in regulatory clusters in the genome, called cis-regulatory modules (CRMs). Some CRMs can repress transcription, whereas others, referred to as ‘enhancers’, can enhance gene expression. Studies in Drosophila showed that the combination of binding sites together with the set of transcription factors actively recruited to a CRM determines its cell-type-specificity (Goto et al., 1989; Small et al., 1991; Zinzen et al., 2009). More generally speaking, regulatory sequences with a similar binding site content can be expected to drive similar expression patterns. This is analogous to coding sequences, where sequence similarity has been used for many years to estimate functional similarity. The pairwise similarity of coding sequences is usually computed using global (Needleman and Wunsch, 1970) or local (Smith and Waterman, 1981) alignments. This approach works well for sequences which are at least partially alignable, however this is not the case for non-homologous CRMs. The location and orientation of binding sites in CRMs that show similar cell-type-specific activity may differ widely, making it impossible to produce alignments.

Alignment-free methods compare sequences according to their word content, see Vinga and Almeida (2003) for an overview. The initial purpose was to design a fast and accurate measure of pairwise (dis-)similarity that could be used in databases where traditional alignments were too slow (Blaisdell, 1986; Hide et al., 1994; Carpenter et al., 2002). In the meantime, alignment-free methods have been applied in other contexts such as phylogeny (Wu et al., 2009) and motif finding (Gordon et al., 2010). The idea to describe a sequence by its word content directly fits the model of CRMs, where we assume that a similar function is reflected in a similar binding site content.

Word-count-based methods have been used to compare regulatory sequences (Kantorovitz et al., 2007; van Helden, 2004). However, these methods calculate the similarity of sequences based on exact word counts, whereas transcription factor binding sites are generally more flexible patterns. Furthermore, the genomic orientation of CRMs and of the binding sites within is most often unknown, highlighting the need to compare sequences according to the word counts on both strands simultaneously. As an example, the word \(w = \text{CATAAT}\) might be bound by the same transcription factor as...
the words CTTAAT and ATTAGG, the former having one substitution, the latter being on the reverse strand. Exact word comparison methods consider these words dissimilar. More generally, let \( n(w) \) be the set of words which are similar to \( w \) (the ‘neighbourhood’ of \( w \)). To overcome the limitation of exact word comparison methods, we need to develop a similarity measure that compares sequences based on word neighbourhoods. Theoretical approaches that consider approximate word matches have been studied before (Burden et al., 2008; Fort et al., 2006), however no applicable method has been published for the purpose of pairwise comparison.

In this study we define \( N2 \), an alignment-free comparison method that integrates all words in the neighbourhood of \( w \). We compare \( N2 \) to other alignment-free methods on simulated sequences and tissue-specific enhancer sequences identified in vivo in mouse embryos. The code and an executable file of the \( N2 \) similarity and other alignment-free methods presented here is available as part of the open source C++ library SeqAn (Doering et al., 2008).

2 METHOD

2.1 The \( N2 \) similarity score

Traditionally, the idea of alignment-free methods is to compare two sequences \( S_1 \) and \( S_2 \), of length \( l_1 \) and \( l_2 \), based on the numbers of all words \( w \) of length \( k \) over the alphabet \( \Sigma = \{a, c, g, t\} \). Let \( A \) be the set of all such words \( w \) with \( |A| \) being the total number of words (\( 4^k \) in the case of DNA sequences). We associate a sequence \( S \) of length \( l \) with the word count vector

\[
N^S = (N^S_{w_1}, N^S_{w_2}, \ldots, N^S_{w_{|A|}}), \text{ with } (1)
\]

\[
N^S_w = \sum_{i=1}^{l-k+1} 1\{S[i..i+k-1] = w\}. \quad (2)
\]

To overcome the restriction to exact word counts, we extend equation (2) to word neighbourhood counts. We define the set of words in the neighbourhood of the word \( w \) as \( n(w) \). The neighbourhood may be defined appropriately for every sequence, for example, to fit transcription factor binding motifs, to allow for reverse complement word counts or to include mismatches. Integrating neighbourhood counts for every word \( w \) reduces the influence of \( w \) itself. This leads to word counts ‘smoothing’, i.e. inexact words are considered similar, but also to ‘blurring’, since inexact words might not be related. To control for these effects, we associate every word \( w' \) in \( n(w) \) with a weight \( a_{w'} \), which may differ for the considered application. We then compute the weighted word neighbourhood counts \( N_{n(w)} \) for every word \( w \) of the sequence \( S \):

\[
N^{S}_{n(w)} = \sum_{w' \in n(w)} a_{w'} N^S_{w'}. \quad (3)
\]

Depending on the choice of \( n(w) \), \( N^S_{n(w)} \) might be the sum of highly dependent variables since word occurrences of overlapping words such as \( AAAAAA \) and \( CAGCTG \) are strongly correlated. Additionally, the variance of individual word counts should be considered, since, for example, a high number of \( CAGCTG \) occurrences is more informative than a high count of self-overlapping words such as \( AAAAAA \) where a Poly-A stretch of length 15 already gives 10 occurrences. Furthermore, some words are more likely to occur than others, \( GC \)-rich words for example are less frequent in mammalian genomes than \( AT \)-rich words. We correct for inter-variable dependency, word count variances and word probabilities by standardising the word neighbourhood counts:

\[
\hat{N}^S_{w} = \frac{N^S_{n(w)} - E[N^S_{n(w)}]}{\sqrt{V[N^S_{n(w)}]}}. \quad (4)
\]

Since the word counts might be dependent, the covariance of all words in the word neighbourhood has to be computed to obtain \( V[N^S_{n(w)}] \) (see 2.2).

We now calculate the normalised standardised neighbourhood count vector \( \hat{N}^S \) as

\[
\hat{N}^S = \left( \hat{N}^S_{w_1}, \ldots, \hat{N}^S_{w_{|A|}} \right)
\]

where \( \hat{N}^S \) represents the Euclidean norm. We define the \( N2 \) similarity of two sequences as the inner product of their normalised standardised word neighbourhood count vectors:

\[
N2(S_1, S_2) = \langle \hat{N}^S_{S_1}, \hat{N}^S_{S_2} \rangle = \sum_{w \in A} \hat{N}^S_{S_1,w} \hat{N}^S_{S_2,w}. \quad (5)
\]

As a consequence of the normalisation, \(-1 \leq N2(S_1, S_2) \leq 1 \), and \( S_1 = S_2 \Leftrightarrow N2(S_1, S_2) = 1 \), i.e. equal sequences will always have the maximum pairwise similarity of 1.

2.2 Calculation of expected value and variance

The \( N2 \) score can be computed with Markov models of any order. Here, we illustrate the calculation of the expected value \( E[N^S_{n(w)}] \) and variance \( V[N^S_{n(w)}] \) assuming a first order Markov model. For clarity, the superscript indicator for sequence \( S \) is omitted in the following. Let the sequences be modelled by a first-order homogeneous stationary Markov chain with transition probabilities \( \pi(i, j) \) (Robin et al., 2005). The probability \( \mu(w) \) that a word \( w \) occurs at a specific position \( i \) depends on the probability that the first letter occurs, denoted \( \mu(w[1]) \) (stationarity of the Markov chain) and can be calculated as follows:

\[
\mu(w) = \mu(w[1]) \times \prod_{j=2}^{k} \pi(w[j-1], w[j]).
\]

With this at hand, we can calculate the expected value \( E[N^S_{n(w)}] \) of the word neighbourhood counts (Robin et al., 2005):

\[
E[N^S_{n(w)}] = E \left[ \sum_{w' \in n(w)} a_{w'} N^S_{w'} \right] = \sum_{w' \in n(w)} a_{w'} E[N^S_{w'}], \text{ with }
\]

\[
E[N^S_{w'}] = (1-k+1)\mu(w').
\]

The variance is important to correct for the dependency of overlapping words in the word neighbourhood. The variance \( V[N^S_{n(w)}] \) of the word neighbourhood counts corresponds to the variance of the sum of the weighted word counts \( N^S_{w'} \):

\[
V[N^S_{n(w)}] = V \left[ \sum_{w' \in n(w)} a_{w'} N^S_{w'} \right] = \sum_{w' \in n(w)} \sum_{w'' \in n(w)} a_{w'} a_{w''} \text{ Cov}[N^S_{w'}, N^S_{w''}].
\]
The covariance of word counts can be calculated according to Robi rate al. (2005):

$$\text{Cov}[N_{w}, N_{w'}] = \mu(w) \sum_{d=1}^{k-1} (l-k-d+1)$$

(5)

$$\mu(w) \sum_{d=1}^{k-1} (l-k-d+1) \left( \epsilon_{k-d}(w, w') \prod_{j=k-d+1}^{k} \left( \pi(w[j-1], w'[j]) - \mu(w') \right) \right)$$

(6)

$$+ \mu(w') \sum_{d=1}^{k-1} (l-k-d+1) \left( \epsilon_{k-d}(w', w) \prod_{j=k-d+1}^{k} \left( \pi(w[j-1], w[j]) - \mu(w) \right) \right)$$

(7)

by which the variance is equal to the expected value.

In the following, we will refer to these instances as $N^{2rc}$, $N^{2mm}$, $N^{2mm-rc}$. The word count of $w$ (and its reverse complement) is always weighted with $\alpha_{w_{0}} = 1$, for all other words $w'$ in $n(w)$ an alternative weight $\alpha_{w'}$ may be chosen. The weights for mismatch neighbourhood counts are indicated in superscript, we use $\alpha_{w_{0}} = 1$ ($N^{2mm-rc}$) if not stated otherwise. Note that in equations (19) and (20) our neighbourhood definition only covers direct neighbours, not neighbours of neighbours.

2.4 Other methods

The simplest score between two sequences $S_{1}$ and $S_{2}$ is obtained by calculating either the euclidean distance (Blaisdell, 1986) or the inner product (Lippert et al., 2002) of the word count vectors $N^{S_{1}}$ and $N^{S_{2}}$ as defined in equation (1). Both methods are called $D2$ and have been applied to biological data (Hude et al., 1994; Carpenter et al., 2002). Here we focus on the latter version using the inner product:

$$D2(S_{1}, S_{2}) = \sum_{w \in A} N^{S_{1}}_{w} \times N^{S_{2}}_{w}$$

$D2$ is directly dependent on the length of the sequences, it can therefore not be used for comparing sequences of different length. The $D2$ z-score ($D2z$) was proposed to obtain a standardised $D2$ score for which the significance can be estimated (Kantorovitz et al., 2007):

$$D2z(S_{1}, S_{2}) = \frac{D2(S_{1}, S_{2}) - \mathbb{E}[D2(S_{1}, S_{2})]}{\sqrt{\mathbb{V}[D2(S_{1}, S_{2})]}}$$

The expected value for $D2$ has been studied for approximate word matches, and upper and lower bounds for the variance have been calculated (Burden et al., 2008). This work is largely of theoretical nature for Bernoulli background models and no implementation is provided, therefore we could not integrate this work into the analysis (see Discussion).

The $D2^*$ score (Reinert et al., 2009) standardises the word counts instead of the inner product. Similarly to $N2$, $D2^*$ is defined as the inner product of the standardised word counts as shown in equation (3), but in this case $n(w)$ only contains $w$ itself, and the background model is computed on the concatenation of both sequences.

Let $\mu(w)$ be the probability of $w$, the expectation of $N^{S}_{w}$ is then estimated by $\mathbb{E}[N^{S}_{w}] = (l-k+1)\mu(w)$. The authors assume a Poisson distribution, which implies that the variance is equal to the expected value. $D2^*$ was originally proposed with a Bernoulli background model for the computation of $\mu(w)$. Here, we extended this score to use Markov background models of higher order. For the purpose of pairwise comparison, the $D2$, $D2^*$ and $D2z$ scores have been implemented in the SeqAn library (Doering et al., 2008) and are part of the executables that is available online.
2.5 Estimating the background Markov model

Calculation of the expected value and variance of the word counts assumes that we know the background model that describes the sequence. For N2, we estimate the background model separately for every sequence. This allows us to precompute word probabilities and variances (see 2.3) leading to a great reduction in computational costs. Since CpG dinucleotides in mammalian genomic sequences are very rare (Gardiner-Garden and Frommer, 1987), a Bernoulli background model is insufficient to estimate word probabilities. This can be seen on simulations, where the first order Markov model consistently outperforms the Bernoulli model across all methods (Supplementary Table 1). The optimal order for the Markov background model for enhancer sequences is an unknown function of organism complexity and sequence length. Due to the limited size of enhancer sequences, estimating higher order Markov models likely results in overfitting and poor estimates. Our analysis will therefore rely on a first order Markov chain as background model for all methods throughout this analysis.

2.6 Masking repeats

Repeats such as SINE elements have a substantial influence on pairwise scores. We use the UCSC pre-masked genome sequence (hg19, RepeatMasker (www.repeatmasker.org), TandemRepeatsFinder(Benson, 1999)) in order to hide those repetitive elements. Any repeat-masked sequence is split into a set of repeat-free sub-sequences by cutting out all repeat regions. Words are counted in this set such that no artifical words are created by concatenating. We use (number of counted words) + k – 1 as an estimation of the length of the repeat-free sequence. Repeat-masked sequences are treated equally for all methods. Note that this is slightly different to the original method proposed for D2z, which introduced artifical words by concatenating sequences.

3 RESULTS

3.1 N2 can be computed quickly

Genome-wide data sets consist of many thousand regulatory sequences. The computation of pairwise similarities needs to be efficient for large-scale usage. We estimated the running time of each score on sets with various numbers of sequences where we computed the matrix of all pairwise similarities (quadratic number of scores computed). The methods show strong differences in practise (Figure 1), but N2 and its variants are always faster than the other scores with a statistical model for realistically chosen numbers. Computing pairwise scores for 5000 enhancers with k = 6 takes 2 hours (h) for N2* (4h for N2 rec, 20h for N2 rec,m), it takes about 42 h for D2z and 91 h for D2.

The computation of N2 is dominated by the pre-processing step which scales linearly in the number of sequences since the neighbourhood counts are calculated once for every sequence in advance (Fig. 1, Table 1, see Methods). In contrast, D2z and D2* cannot precompute normalised counts like N2, and scale quadratically in the number of sequences. D2z calculates z-scores on pairs of sequences which are not preprocessed (Kantorovitz et al., 2007), and D2* calculates the background model on the concatenation of sequences which cannot be precomputed (Reinert et al., 2009). While this is likely to increase the accuracy of the model, running times are drastically higher. Computing pairwise scores for realistically large data sets is therefore nearly impossible for both D2z and D2*. This makes the N2 score very attractive for large-scale applications such as classification of regulatory sequences, or applications that support precomputed data structures such as database searches.

3.2 N2 is robust against single sequence noise

Ideally, the pairwise score between two sequences should reflect the sequences’ similarity. However, in practise, word-count-based methods can be heavily influenced by noise specific to individual sequences, meaning that some sequences will intrinsically have high (or low) scores (Lippert et al., 2002; Reinert et al., 2009). Without proper correction, the pairwise score is an attribute of the individual sequence rather than of the pair of sequences. This is especially prominent for D2, where a high number of occurrences of a repetitive self-overlapping word (such as AAAAAA) in one sequence will always induce high pairwise scores. To quantify the influence of single-sequence-specific noise on pairwise scores, we studied the behaviour of D2, D2z, D2 and N2 for scoring pairs of unrelated sequences simulated by the same background model. We calculated scores for all sequence pairs (Si, Sj) for 500 such unrelated sequences. We chose a threshold t to select the top 5% highest scoring sequence pairs (‘high scoring pairs’). For every sequence Si, we calculated the number of high scoring pairs’ Ci: $C_i = \sum 1(\text{score}(S_i, S_j) \geq t)$. Since all sequences were generated by the same model, the expected value of $C_i$, $E(C_i)$, is equal for all sequences $S_i$. Here, 5% of the 499 sequence pairs of $S_i$ are expected to have a score greater than $t$, thus $E(C_i) = 24.95$. As reference, we calculated $C = \{ C_1, ..., C_i \}$ when we randomly assign scores.
to sequence pairs. This method is not influenced by the sequence at all and therefore recapitulates the expected behaviour for unrelated sequence pairs (Figure 2, black line). We then calculated $C_i$ for the four alignment free sequence comparison methods.

The distribution of $C$ when $N^2$ is used is close to the expected distribution for unrelated sequences (Figure 2). This shows that $N^2$ is robust against single-sequence-specific noise as the number of high scoring pairs is not influenced by the individual sequences (see Supplementary Figures 1 and 2 for dual sequences). In contrast, $D2$ and $D2z$ show a very different distribution of $C$ from the expected behaviour in the non-uniform case. Figure 2B shows that the number of high scoring pairs strongly varies, suggesting that the expected number for $C_i$ is different for every sequence $S_i$, even though all sequences were generated by the same model. This shows that the number of high scoring pairs detected with these methods is strongly influenced by the individual sequence, indicating that pairwise scores measure the individual sequence composition and not the similarity of the sequence pair. Prior work comparing regulatory sequences using alignment-free methods did not consider this effect (Kantorovitz et al., 2007; Dai et al., 2008). The above results confirm that neither the $D2$ nor the $D2z$ score should be applied to real biological sequences (Lippert et al., 2002; Reinert et al., 2009).

2. Influence of sequence composition on pairwise scores for 500 sequences generated by the same model were calculated. $C_i$ measures the number of sequence pairs for sequence $S_i$ among the highest 5% of all scores (‘high scoring pairs’). Since all sequences were generated using the same model, the distribution of $C = (C_1, \ldots, C_N)$ from alignment-free methods should be similar to the distribution of $C$ obtained from a random scoring method (‘expected’, black line). A different distribution would indicate that the number of high scoring pairs is strongly dependent on the individual sequence, indicating that pairwise scores are dependent on the single sequence noise rather than on the similarity of the sequence pair. (A) Uniform nucleotide distribution, all methods show the expected behaviour. (B) AT rich nucleotide distribution, $D2$ and $D2z$ differ from the expected behaviour, showing that these pairwise scores are strongly influenced by the sequence composition.

3.3 Simulation studies

To test the performance of $N^2$ on simulated data, we randomly generated sequences with a similar dinucleotide content as the mouse genome (Thomas-Chollier et al., 2011) (mm9) as background sequences (‘negative set’). We then implanted $m$ randomly chosen motifs of length $r$ times into the same background sequences to simulate CRMs (‘positive set’; $m18$: $m = 1$, $r = 8$; $m4r2$: $m = 4$, $r = 2$). Following Kantorovitz et al. (2007), we computed all pairwise scores for the corresponding negative and positive sets. The pairwise scores from the negative and the positive sets were combined and ranked. Based on this ranked list, we evaluated the performance of the above methods for pairwise sequence comparison using the area under ROC curve (AUC ROC) and area under Precision-Recall curve (AUC PR). We further estimated the interpolated precision at 5% recall which we term 5%-Precision for short. Results show average values over 25 simulations, each time drawing 100 random sequences of length 1000 bp and inserting random motifs, thus covering different motif compositions in an unbiased way. We tested the performance counting words of size $k = 6$ using a first order Markov model for word probabilities (see Supplementary Tables 3 and 4 for $k = 5$).

We simulated two different settings to evaluate the performance of the neighbourhood concept of $N^2$. First, we implanted randomly sampled 5-mers into the forward and backward strand of the sequences to simulate the orientation independence of binding sites in CRMs. We specifically designed the $N^2$ variant for this

### Table 2: Comparison of the different methods ($k = 6$) when the genomic orientation of the motif is unknown. Bold numbers indicate best performance.

<table>
<thead>
<tr>
<th>Performance with implanted k-mers, random strand</th>
<th>5%-Precision</th>
<th>AUC ROC</th>
<th>AUC PR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motif setting: m18 m4r2 m4r2 m18 m18 m4r2 m4r2 m4r2</td>
<td>D2</td>
<td>0.88</td>
<td>0.59</td>
</tr>
<tr>
<td>D2z</td>
<td>0.91</td>
<td>0.64</td>
<td>0.74</td>
</tr>
<tr>
<td>D2$^*$</td>
<td>0.87</td>
<td>0.66</td>
<td>0.71</td>
</tr>
<tr>
<td>N$^2$</td>
<td>0.86</td>
<td>0.65</td>
<td>0.71</td>
</tr>
<tr>
<td>N$^2$$^{rc}$</td>
<td>0.93</td>
<td>0.71</td>
<td>0.77</td>
</tr>
</tbody>
</table>

### Table 3: Comparison of the different methods ($k = 6$) when motifs are sampled from all k-mers with one mismatch to the word. Bold numbers indicate best performance.

<table>
<thead>
<tr>
<th>Performance with implanted k-mers, mismatch</th>
<th>5%-Precision</th>
<th>AUC ROC</th>
<th>AUC PR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motif setting: m18 m4r2 m4r2 m18 m18 m4r2 m4r2 m4r2</td>
<td>D2</td>
<td>0.59</td>
<td>0.54</td>
</tr>
<tr>
<td>D2z</td>
<td>0.59</td>
<td>0.54</td>
<td>0.54</td>
</tr>
<tr>
<td>D2$^*$</td>
<td>0.60</td>
<td>0.54</td>
<td>0.54</td>
</tr>
<tr>
<td>N$^2$</td>
<td>0.59</td>
<td>0.54</td>
<td>0.54</td>
</tr>
<tr>
<td>N$^2$$^{mm}(0.01)$</td>
<td>0.60</td>
<td>0.54</td>
<td>0.55</td>
</tr>
<tr>
<td>N$^2$$^{mm}(1.0)$</td>
<td>0.65</td>
<td>0.55</td>
<td>0.57</td>
</tr>
</tbody>
</table>
ability of alignment-free sequence comparison methods to detect similar activity from genomic background. We used enhancers active in forebrain, midbrain, limb and heart tissue of the developing mouse embryo as positive sets (Visel et al., 2009; Blow et al., 2010). These data sets allow us to test whether alignment-free methods are able to discriminate in-vivo identified enhancers that show similar activity from genomic background. We used enhancers active in forebrain, midbrain, limb and heart tissue of the developing mouse embryo as positive sets (Visel et al., 2009; Blow et al., 2010). We compared pairwise scores from these tissue-specific enhancers with pairwise scores from genomic sequences of the same length randomly sampled from the mouse genome, ensuring a maximum of 30% of repetitive sequence for every negative sample. To obtain accurate estimations, we calculated the average over 25 samples, each time drawing 500 sequences from the positive set. Using the functional similarity of regulatory sequences.

3.4 Pairwise comparison of tissue-specific enhancers

The above simulations demonstrated the ability of N2 to distinguish artificial CRMs from unrelated sequences. Currently, our knowledge on regulatory sequences is limited and simulations can only approximate the real nature of enhancers. Tissue-specific enhancers in mouse embryos have been identified in a genome-wide manner using the co-activator protein p300 (Visel et al., 2009; Blow et al., 2010). These data sets allow us to test whether alignment-free methods are able to discriminate in-vivo identified enhancers that show similar activity from genomic background. We used enhancers active in forebrain, midbrain, limb and heart tissue of the developing mouse embryo as positive sets (Visel et al., 2009; Blow et al., 2010). We compared pairwise scores from these tissue-specific enhancers with pairwise scores from genomic sequences of the same length randomly sampled from the mouse genome, ensuring a maximum of 30% of repetitive sequence for every negative sample. To obtain accurate estimations, we calculated the average over 25 samples, each time drawing 500 sequences from the positive set. Using the functional similarity of regulatory sequences.

The choice of parameters will influence the results obtained from alignment-free comparisons. For N2, the main parameters are the length of the k-mers k and the weights of the words in the neighbourhood (a_w). We therefore tested k = 4, 5, 6 and mismatch weights a_w = \{1, 0.75, 0.5, 0.25, 0.1, 0.05, 0.01, 0.001\} (Supplementary Figures 3-4). These simulations confirm the value of extending exact word count methods to word neighbourhoods.
measure similarity of allows us to verify and increase our understanding of the architectures based on shared words. Importantly, This suggests that there are subtle signals like a common content improves the performance on experimentally identified enhancers. The differences between \((N2^z)\) is better than the improvement due to different \((D2^z)\) further improves the performance on tissue-specific enhancer sequences. This assumption is violated by repeats, having a high number of shared words only due to high sequence similarity. For this reason we mask repeats before calculating pairwise scores. Although some transcription factor binding sites have been found in repetitive sequences (Kunarso et al., 2010; Zemojtel et al., 2009), the sequence similarity of repeats is largely unrelated to regulatory activity and will eclipse any shared word count from common DNA binding motifs. We therefore recommend the usage of repeat masked sequences when comparing regulatory elements.

The \(N2\) similarity can be applied to other tasks than pairwise comparison. Alignment-free methods have been used to predict cis-regulatory modules in flies and mouse (Kantorovitz et al., 2009). Our results on pairwise comparison of enhancers suggest that the \(N2\) similarity could as well be used to predict the regulatory outcome of enhancers. In contrast to pairwise comparison, where we only rely on two sequences, prediction would allow to use training data, therefore we expect that the performance would improve for this task. Nevertheless the large size of mammalian genomes limits prediction of regulatory sequences in a genome-wide manner due to an inevitable large number of false positive predictions. Among the applications where \(N2\) might be very insightful are clustering and classification of regulatory sequences obtained from genome-wide studies using transcription factor or co-activator binding data (Lee et al., 2011), DNase hypersensitivity sites or enhancer specific histone modifications.

### 5 CONCLUSION

In this work we have presented \(N2\), a novel alignment-free measure of sequence similarity that overcomes the limitations imposed by traditional exact-word-count based methods. We have included the general concept of weighted word neighbourhood counts and shown that it improves the ability to detect similarity between regulatory sequences. The task of pairwise comparison of regulatory sequences is much harder than traditional pairwise alignment since only very few shared words might lead to a similar activity. We have demonstrated on a large-scale data set of mammalian enhancer sequences that pairwise sequence similarity of non-homologous regulatory sequences is able to estimate similar \(in vivo\) activity. We are therefore getting closer to understanding the...
sequence-dependent regulatory code within CRMs that enables the establishment of a large diversity of cell types coded in one genomic sequence.

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