A Machine Learning Strategy to Identity Exonic Splice Enhancers in Human Protein-coding Sequence

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Abstract

Background: Exonic splice enhancers are sequences embedded within exons which promote and regulate the splicing of the transcript in which they are located. A class of exonic splice enhancers are the SR proteins, which are thought to mediate interactions between splicing factors bound to the 5' and 3' splice sites.

Method and results: We present a novel strategy for analysing protein-coding sequence by first randomizing the codons used at each position within the coding sequence, then applying a motif-based machine learning algorithm to compare the true and randomized sequences. This strategy identified a collection of motifs which can successfully discriminate between real and randomized coding sequence, including – but not restricted to – several previously reported splice enhancer elements. As well as successfully distinguishing coding exons from randomized sequences, we show that our model is able to recognize non-coding exons.

Conclusions: Our strategy succeeded in detecting signals in coding exons which seem to be orthogonal to the sequences’ primary function of coding for proteins. We believe that many of the motifs detected here may represent binding sites for previously unrecognized proteins which influence RNA splicing. We hope that this development will lead to improved knowledge of exonic splice enhancers, and new developments in the field of computational gene prediction.

1 Introduction

Alternative splicing is a major mechanism of diversity in the expression of eukaryotic genes, and has also been implicated in gene regulation (Modrek and Lee, 2002; Brett et al, 2000; Graveley, 2001; Harrison et al, 2002; Lewis et al, 2003). A number of sequences have been found embedded in the exons of both viral and cellular genes which can promote or repress the utilization of alternative splice sites. Exonic splice enhancers are usually purine-rich sequences located in an alternatively splice donor. Through specific binding of proteins, including the serine/arginine-rich SR family, exonic splice enhancers function by recruiting splicing factors such as U2AF
to a suboptimal splice acceptor in the early stages of spliceosome assembly, thereby stimulating splicing of the upstream intron or inclusion of the alternative exon.

SR proteins are a family of highly conserved serine/arginine-rich RNA-binding proteins (For review, see Graveley 2000). They are essential splicing factors and also regulate the selection and use of alternative splice sites (Bourgeois et al, 1999; Liu et al, 1998; Lynch and Maniatis, 1996; Schaal and Maniatis, 1999; Tacke and Manley, 1995; Tacke et al, 1999; Tian and Kole, 2001; Zheng et al, 1998). It is known that these proteins function very early in the spliceosome assembly process. They promote the binding of U1 snRNP to the splice donor and of U2AF to the splice acceptor, apparently by interacting with U1 70K and U2AF respectively. Observations have shown that SR proteins bound to the exonic splice enhancers recruit splicing factors to the adjacent splice sites. There are nine human SR proteins which are presently known and studied: SF2/ASF (Graveley, 2000; Liu et al, 1998; Schaal and Maniatis, 1999; Tacke and Manley, 1995), SC35 (Liu et al, 1998; Tacke and Manley, 1995; Schaal and Maniatis, 1999; Tacke et al, 1999), SRp20 (Schaal and Maniatis, 1999; Tacke et al, 1999), SRp40 (Liu et al, 1998), SRp55 (Liu et al, 1998), SRp75 (Graveley, 2000), SRp30c (Graveley, 2000), 9G8 (Schaal and Maniatis, 1999; Tian and Kole, 2001) and the divergent SRp54 (Graveley, 2000). These proteins are closely related in sequence and structure and share the ability to activate splicing. Another class of human SR related proteins, the Tra2 family, are also known to be splicing regulators and sequence specific activators of pre-mRNA splicing (Tacke et al, 1999).

Early research concentrated on how SR proteins function to regulate alternative splicing. However, the binding of SR proteins to constitutive exons – those which are included in all splice variants of a gene – also plays an important role in the splicing reaction. The exon definition model proposes that interactions between components bound to splice sites flanking an exon serve to highlight exons – which are usually small – against a background of much larger introns. It is conjectured that the majority of constitutively spliced exons are defined by this mechanism. To support the model, a number of SR protein binding sites have been identified in constitutive exons, and also shown to be constitutive splicing enhancers (Schaal and Maniatis, 1999; Lam and Hertel, 2002).

Although examples of exonic splice enhancers are believed to be common, studying their sequences is difficult because they are embedded in exons, most of which are also functional protein-coding sequences. Non-coding exons are also thought to contain many sequences which are functional in the mature RNA, such as regulators of RNA stability, so the situation there is not necessarily clearer. When a particular motif is found to be over- or under-represented in coding exons, it is generally unclear whether it is a consequence of the underlying protein sequence, or an unrelated signal – such as a splice enhancer – embedded in the protein coding sequence. Here we propose a novel strategy for resolving this uncertainty. Starting with annotated coding exons, we generate a ‘neutralized’ exon set: sequences which are generated randomly, but which nevertheless preserve both the amino acid sequence and overall composition features of the true exons. We then apply machine learning software to compare the true and neutralized exons. Since the neutralized set codes for the same
proteins, it is likely that any feature which can be used to discriminate between the true and neutralized sets is performing some function which is independent of the exons’ primary, protein-coding, function.

The neutralization process we use has some similarities to the dicodon shuffling algorithm proposed by Katz and Burge (2003), which swaps pairs of synonymous codons under a constraint that the dinucleotide composition of the sequence must be preserved. However, our method differs both in implementation strategy and in the fact that dinucleotide composition is maintained across the complete set of sequences, rather than on a per-sequence basis (see results in section 2.1).

An alternative, very different, computational method for finding splice enhancer signals has recently been proposed: RESCUE-ESE (Fairbrother et al, 2002) compares the sequences around weak consensus splice sites with those around strong consensus sites, with the expectation that splice enhancer motifs are more likely to be found in the vicinity of weak splice sites. This strategy is very different from ours, and so it is interesting to compare the results.

2 Results

2.1 Neutralized exons

Internal coding exons with lengths ranging from 100 to 300 bases were extracted from the Vega database of annotated human genomic sequence [http://vega.sanger.ac.uk/]. Testing the neutralization process on a typical 300 base exon (figure 1) we see that the level of sequence identity falls steadily for approximately 500 cycles, then comes close to its minimum value and only fluctuates slightly for the remainder of the cycles. Allowing some margin for exceptional sequences, this suggests that 1000 cycles of neutralization is adequate to randomize any sequence with a length up to 300 bases.

Running the neutralization algorithm on the complete set of qualifying exons, for 1000 cycles per exon, gave a neutralized set of with an average of 78% identity. The average dinucleotide compositions of the exons before and after neutralization is shown in table 1. We can see that most dinucleotides show negligible change in composition during the neutralization procedure, and in the most extreme case (the tt dinucleotide), the proportion of the sequences composed of tt dinucleotides changes by less that 2%. Therefore, we consider the neutralization algorithm to be successful in preserving overall sequence composition while substantially changing the sequence itself. On the same sequences, the dicodon shuffling algorithm typically gives a sequence identity of around 90%.

2.2 Motif-based models can effectively distinguish between true and neutralized exons

Our data set consisted of 9091 true coding exons ranging in length from 100 to 300 bps, and an equal number of neutralized counterparts (see methods section). From
Figure 1: Time-course for neutralizing a typical 300 base sequence.

| Dimucleotide | True exons | Neutralized exons |
|--------------|------------|-------------------|
| aa           | 7.11%      | 7.33%             |
| ac           | 5.56%      | 5.55%             |
| ag           | 8.19%      | 8.21%             |
| at           | 5.58%      | 5.62%             |
| ca           | 8.06%      | 8.06%             |
| cc           | 7.17%      | 7.18%             |
| cg           | 2.73%      | 2.74%             |
| ct           | 6.96%      | 6.88%             |
| ga           | 7.76%      | 7.76%             |
| gc           | 6.41%      | 6.38%             |
| gg           | 6.61%      | 6.54%             |
| gt           | 4.55%      | 4.58%             |
| ta           | 3.43%      | 3.47%             |
| tc           | 5.72%      | 5.69%             |
| tg           | 7.98%      | 7.96%             |
| tt           | 5.48%      | 5.58%             |

Table 1: Comparison of dimucleotide frequencies in true and neutralized exons
both the true and neutralized sets, we removed 300 randomly selected sequences for use as test data. The remainder were used to train a Convolved Eponine Windowed Sequence (C-EWS) model (see methods and Down and Hubbard 2003). These models are based on scaffolds of one of more sequence motifs (in this case, limited to a maximum of three per scaffold). The motifs are represented as DNA weight matrices (Bucher, 1990). When a scaffold includes more than one motif, probability distributions associated with each motif indicate the preferred relative positions of those motifs. Each scaffold has an associated weight, which is used to combine scaffold scores in a generalized linear model.

This training procedure resulted in a complex model consisting of 216 scaffolds, split evenly between positively-weighted scaffolds – signals which are likely to be over-represented in the true exons – and negatively weighted scaffolds. The complete set of scaffolds can be seen in figures 2 and 3.

We tested the resulting model’s classification ability using the unseen data. Accuracy (specificity or proportion of positive predictions which are correct) and coverage (sensitivity or proportion of true exons detected) are shown for a range of classifier score thresholds in figure 4. Clearly, the features learned by our procedure are effective, in the general case, for distinguishing between true and neutralized sequences.

Figure 2: Positively weighted scaffolds in the Eponine Exons model
2.3 The eponine-exon model can also distinguish non-coding exons from randomized sequences

Since we used semi-random sequences as the negative training set, an obvious concern is that the features we have detected are artifacts of the neutralization procedure, and are of no use when analysing real sequence data. To validate the Eponine exon model, we tested it on additional sequences from four classes: coding exons, non-coding (UTR) exons, introns, and intergenic regions, all according to Vega annotation of finished human chromosomes. In each case, we obtained a set of 1200 example sequences, each of 200 bases long. For intergenic regions, we obtained four independent sets of 1200 sequences.

For each data set, we produced a corresponding set of negative sequences with
matching mono- and di-nucleotide composition using the randomizing procedure detailed in the methods section 4.4. We then used the Eponine-Exons model as a classifier, and tested its ability to separate each of the positive sequence sets from its corresponding negative sequence set. Receiver Operating Characteristic curves are shown in figure 5.

In the case of the intergenic sequences, there is no significant discrimination between real and shuffled sequences. The coding sequences, however, could be discriminated, as might be expected from a classifier trained on protein-coding sequences. However, the model was also able to distinguish many non-coding exons from their shuffled counterparts. This result is highly significant because it indicates that at least some of the signals discovered in coding exons are actually general to both coding and non-coding exons – consistent with the idea that they are involved in exon definition and splicing. Finally, there is a far weaker, but still possibility significant, discrimination of introns. One explanation for this is that the introns were contaminated with a small number of exons which were missed during the annotation process. However, a second possibility is that, in addition to an exon-specific signal, the Eponine Exons model is also detecting some (weak) signal – perhaps an anti-termination signal – which is found throughout transcribed regions of the genome.

Figure 4: Accuracy vs. coverage testing the model’s ability to discriminate between unseen true and neutralized exons.
Figure 5: ROC curves for the Eponine-Exons model on intergenic, intron, and UTR exon sequences compared with random sequences of matching mono- and di-nucleotide composition. All curves are based on sets of 1200 sequences. In the case of intergenic sequences, standard-deviation error bars were calculated based on results from four independent sets of sequences.

2.4 Comparison of learned motifs with known splice-enhancer sites

We compared the weight matrices in the positively weighted scaffolds of our exon model with known splice enhancer sites (Graveley, 2000; Bourgeois et al, 1999; Liu et al, 1998; Lynch and Maniatis, 1996; Schaal and Maniatis, 1999; Tacke and Manley, 1995; Tacke et al, 1999; Tian and Kole, 2001; Zheng et al, 1998), and also motifs detected by a very different computational approach, RESCUE-ESE (Fairbrother et al, 2002). Direct comparisons of weight matrices with sequence motifs – with or without ambiguity symbols – is complicated, since different positions in a weight matrix may convey different amounts of information. Furthermore, it is not certain that either the learned weight matrices or the published motifs correspond to the full length of the biologically functional sequence. It it therefore important to consider a range of possible alignments of motifs to weight matrices.

For each motif, we calculated the log-odds score against all weight matrices from scaffolds with weights greater than 1.0, considering all possible alignments with up to one base of overhang, and took the maximum score. We then generated 500 shuffled variants of the motif and scored these in the same fashion, taking the mean to be a
representative score for motifs of that particular length and base composition. Tables 2, 3 and 4 lists the direct and shuffled scores for experimentally determined motifs with our training model, and also the difference between them. The status in the tables is to score a "+" or a "-" if the absolute observed difference is greater than 0.2. Our model has managed to predict some of the consensus sequences for exonic splicing enhancers that are located within the internal exon (see table 2) and did not detect any exonic splicing enhancers consensus in the intronic regions near splice acceptor sites (see table 3). The Eponine-Exons model successfully distinguished the intronic and exonic consensus sequences for exonic splicing enhancers. Our model also finds some, but not all, of the motifs detected by the RESCUE-ESE method. This is consistent with the result above, since RESCUE-ESE is designed to detect both exon-localized and intron-localized motifs.

An unusual feature of our learning system is its ability to capture scaffolds of related motifs, as well as individual motifs. The scaffold (aagaatga aegc cccg) is particularly noteworthy. It is identified with the two known exonic splice enhancers, ASF/SF2 and SC35, which are known to possess distinct, functionally significant RNA binding specificities (Tacke and Manley, 1995). In addition, the SR-related protein binding site for the Tra2β is also recognized for the motif aagaatga. The mammalian Tra2 proteins are shown to be sequence-specific activators of pre-mRNA splicing. The scaffold suggests that there might be a connection between Tra2 proteins with the splice enhancers ASF/SF2 and SC35. This might provide a starting point for predicting the relationship between exonic splice enhancers and sequence specific activators.

3 Discussion

We have shown that a motif-oriented machine learning strategy can extract signals which discriminate effectively between true and neutralized sets of coding exons. The resulting model included recognizable consensus sequences for many of the previously reported splice-enhancer binding sites. Although the model was trained only on coding exon sequences, it gives high scores for both coding and non-coding exons, but not introns or intergenic regions. We therefore believe that the neutralization strategy is a powerful and effective method for learning functional non-coding elements embedded in protein coding sequence.

One interesting feature of the model learned here is its complexity: 216 scaffolds, split evenly between positively and negatively-weighted scaffolds. This is a large number, both in absolute terms, and also in comparison with EWS and C-EWS models trained for other purposes, such as promoter prediction (T. Down, unpublished). This suggests that a large number of functional elements play widespread roles in exon definition. Those motifs learned here which cannot be assigned to any currently known splice-regulating protein are strong candidates for investigation with a view to discovering novel splice regulators. It may also be worth further investigation of the combination of motifs which appear in scaffolds, since this could indicate interactions between proteins in the splicing complex.
| Consensus  | SR Protein | Direct Score | Shuffled Score | Difference | Status | Reference            |
|-----------|------------|--------------|----------------|------------|--------|----------------------|
| aggacagagc| ASF/SF2    | 3.823        | 3.464          | 0.3589     | +      | Tacke et al (1995)   |
| aggacgaagc| ASF/SF2    | 3.823        | 3.449          | 0.3737     | +      | Tacke et al (1995)   |
| rgaagaac  | ASF/SF2    | 3.207        | 3.296          | -0.088     |        | Tacke et al (1995)   |
| acgcgca   | ASF/SF2    | 3.222        | 2.962          | 0.2602     | +      | Tacke et al (1995)   |
| aggacrragc| ASF/SF2    | 3.823        | 3.267          | 0.5564     | +      | Graveley (2000)      |
| tscgkm    | SRp55      | 2.860        | 2.568          | 0.2915     | +      | Liu et al (1998)     |
| cctcgcccc | SRp20      | 3.209        | 2.967          | 0.2420     | +      | Tacke et al (1999)   |
| tgttcsagwt| SC35       | 3.484        | 2.877          | 0.6063     | +      | Tacke et al (1999)   |
| tgcngyy   | SC35       | 2.748        | 2.622          | 0.1262     |        | Schaal et al (1999)  |
| acgaggay  | 9G8        | 3.508        | 3.137          | 0.3709     | +      | Graveley (2000)      |
| tcwwc     | dsx        | 2.553        | 2.146          | 0.4070     | +      | Schaal et al (1999)  |
| aggagat   | SC35       | 3.823        | 3.269          | 0.5546     | +      | Graveley (2000)      |

Table 2: Comparison of known ESE motifs which are located inside the internal exons. The status in the tables is to score a "+" or a "-" if the absolute observed difference is greater than 0.2.

We hope that changes in the machine learning strategy will improve the classification accuracy of this method. Possible candidates for investigation include the use of scaffolds comprising more than 3 motifs, and the replacement of simple weight matrices with more complex models which serve as better representations of protein binding sites. We do not, however, necessarily expect that it will be possible to classify true and neutralized exons with 100% accuracy: most proteins can accept many mutations with little or no change to structure and function, so it is inevitable that some of the information which the cell uses to define exons will be encoded in the choice of amino acids, rather than just the choice of nucleotides used in redundant positions.

In the future, we hope to apply the results of this technique to the problem of ab initio prediction of genes. Current gene-prediction techniques rely on a combination
| Consensus | SR Protein | Direct Score | Shuffled Score | Difference | Status | Reference          |
|-----------|------------|--------------|----------------|------------|--------|--------------------|
| ctkctcy   | SRp20      | 2.507        | 2.410          | 0.0976     | -      | Schaal et al (1999) |
| rgaccgg   | SC35       | 3.064        | 3.065          | -0.001     | -      | Schaal et al (1999) |
| ggacaa    | ASF/SF2    | 2.507        | 3.096          | -0.588     | -      | Schaal et al (1999) |
| ggacag    | ASF/SF2    | 2.507        | 3.049          | -0.541     | -      | Schaal et al (1999) |
| agagcagg  | ASF/SF2    | 2.405        | 3.336          | -0.930     | -      | Zheng et al (1999)  |
| rgackagay | 9G8        | 2.196        | 3.035          | -0.838     | -      | Tian et al (1999)   |
| aagaagaa  | Tra2 (beta)| 3.056        | 3.477          | -0.421     | -      | Tacke et al (1995)  |
| tcaaca    | Tra2       | 2.287        | 2.726          | -0.439     | -      | Lynch et al (1996)  |
| gaagaa    | hTra2 (beta)| 3.056   | 3.393          | -0.337     | -      | Graveley (2000)     |
| gacgacgag | Pu1        | 2.296        | 3.347          | -1.050     | -      | Bourgeois (1999)    |
| gatgaagag | Pu2        | 2.769        | 3.497          | -0.727     | -      | Bourgeois (1999)    |

Table 3: Comparison of known ESE motifs which are located at the splice acceptor site of the exon-intron boundaries

of splice-site models and ‘coding bias’ – the observation that coding sequence looks very different from intronic and intergenic sequence when considering properties such as hexamer frequencies. While such methods work reasonably well for protein-coding genes, they seldom make good predictions of untranslated regions, and do not detect the non-coding RNA genes which are now known to be important in many aspects of cellular function. Scanning bulk genomic DNA using our model makes many predictions outside known exons (i.e. a high apparent false positive rate). This suggests that while the motifs discovered here may be necessary for efficient splicing, they are not sufficient to fully define exons. We hope that building knowledge of splice enhancers into gene prediction methods, together with other features such as splice junction consensus sequence, will improve the prediction of all spliced transcripts, whether coding or non-coding.
| Consensus | Direct Score | Shuffled Score | Difference | Status |
|-----------|--------------|----------------|------------|--------|
| atcttc    | 2.920        | 2.580          | 0.3400     | +      |
| actaca    | 1.982        | 2.729          | -0.746     | -      |
| ttggat    | 3.165        | 2.556          | 0.6090     | +      |
| gaatca    | 3.328        | 3.040          | 0.2883     | +      |
| gaagaa    | 3.056        | 3.416          | -0.360     | -      |
| ttcaga    | 4.600        | 2.850          | 1.7501     | +      |
| gacaaa    | 2.922        | 3.093          | -0.171     |       |
| ctgaag    | 2.769        | 2.931          | -0.162     |       |
| aatcca    | 2.835        | 2.736          | 0.0984     | +      |
| aaccttc   | 3.155        | 2.667          | 0.4877     | +      |

Table 4: Comparison of known ESE motifs with the RESCUE-ESE method in Fairbrother et al (2002)

4 Materials and methods

4.1 Genome sequence and annotation

Human genome sequence release NCBI33 from Ensembl databases (Hubbard et al, 2000). Curated annotation of gene structures on chromosomes 6, 13, 14, 20, and 22 were obtained from the Vertebrate Genome Annotation (Vega) database [http://vega.sanger.ac.uk]. We extracted a total of 27954 internal translated coding exons (see definition in Clark and Thanaraj 2002) of different intron phases for our positive training set. Based on the definition (Clark and Thanaraj, 2002), an intron contained within CDS is said to have a phase of zero if the intron demarcates a codon boundary, a phase of one if it divides the codon between the first and second nucleotides, and a phase of two if the intron divides a codon between the second and third nucleotides. The position of an exon with respect to the codon positions can be defined by the phases of upstream and downstream flanking introns and when an exon is flanked by introns of the same phase, it will be a multiple of three nucleotides in length. The phase definition is important for the neutralization scheme described in section 4.3.

Vega and Ensembl data was extracted directly from the SQL databases using the BioJava toolkit with biojava-ensembl extensions [http://www.biojava.org/].

4.2 Constructing a non-redundant set of sequences

To eliminate similar sequences from the datasets, we performed an all-against-all comparison of the sequences using NCBI blastn (Altschul et al., 1999) using default options (word size 11, match reward +1, mismatch penalty −3) and recorded all pairs with a bit score ≥ 35. We then performed single-linkage clustering, and from each cluster we picked one member at random to represent that cluster in the final
4.3 Neutralization of coding sequences

Exon neutralization is a process which randomizes the sequence of a set of protein-coding exons while maintaining three key constraints:

- The neutralized exons code for the same protein sequence as the real exon
- The frequency of a particular codon being used to represent a particular amino acid is maintained
- The overall dinucleotide composition of the set is maintained

Thus, by comparing neutralized exons against the corresponding set of true exons, it should be possible to detect larger sequence features and motifs which are preferentially over- or under-represented in the true exon set. Features which occur purely as artifacts of the underlying protein sequence, or as a result of an overall preference to use particular codons, will occur with equal frequency in the true and neutralized sets.

The neutralization process used here is a Monte-Carlo method, whereby small (single-codon) changes to the sequence are proposed, then accepted or rejected on the basis of a probabilistic model which captures the features listed above. In this case, the model is encapsulated as a set of conditional codon usage tables. Consider a codon $C$ which encode amino acid $A$, and is flanked by nucleotides $p$ and $q$ to form the pentanucleotide $pCq$. Our model records the probability of the codon being used in this context:

$$P(C|A,p,q)$$

The model is initialized for a given set of exons by simply counting all in-frame codons in the exon set. Obviously, this means that a large data set is required to construct a representative model, but the curated human gene set is sufficiently large that this no longer presents a problem

Now, for each exon in the set, a number of neutralization cycles are performed. In each cycle, one in-frame codon position within the exon is chosen at random. Let $C$ equal the current codon at this position. If it encodes an amino acid which has only a single codon in the universal genetic code, it is always left unchanged. Otherwise, a synonymous codon, $C'$, is proposed by sampling from a uniform random distribution over all synonyms, $Q(C'|C)$. Next, the appropriate conditional codon usage table is consulted, given the two bases either side of $C$. We accept or reject the proposed change on the basis of the Metropolis-Hastings criterion:

$$z = \frac{P(C')Q(C'|C)}{P(C)Q(C|C')}$$

$$13$$
When $z \geq 1$, the codon substitution is always accepted, when $z < 1$ the substitution is accepted with probability $z$. In this case, at any given position, the proposal distribution $Q$ is always uniform, the second term of this expression can be ignored: it is simply the fit of the proposed new codon to the model represented by the conditional codon usage tables which is important.

4.4 Generating random sequences with matching mono- and di-nucleotide composition

First, the sequence set is analysed and the initial dinucleotide composition is recorded. We then perform a large number (typically 500) iteration in which two points within the sequence are selected at random, breaking it into three segments, $ABC$. We then propose a rearrangement to give the sequence $BAC$. This rearrangement destroys two dinucleotide pairings and creates two new pairings. The probabilities of the sequences $ABC$ and $BAC$ a calculated from the dinucleotide frequency table, and the rearrangement is accepted or rejected based on the Metropolis-Hastings criterion described above.

4.5 The Eponine Windowed Sequence model

Convolved Eponine Window Sequence (C-EWS) models were trained using a Variational Relevance Vector Machine as described in Down and Hubbard (2003). The model was seeded using five-base motifs. During the training process, sampling rules allowed the motifs to be shortened, lengthened, or combined into scaffolds of up to three motifs.

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