DNA Familial Binding Profiles Made Easy: Comparison of Various Motif Alignment and Clustering Strategies

Shaun Mahony1,2*, Philip E. Auron3,4, Panayiotis V. Benos1,5,6*

1 Department of Computational Biology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America, 2 Department of Computer Science, Faculty of Arts and Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America, 3 Department of Biological Sciences, Duquesne University, Pittsburgh, Pennsylvania, United States of America, 4 Department of Molecular Genetics and Biochemistry, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America, 5 Department of Human Genetics, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America, 6 University of Pittsburgh Cancer Institute, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America

Transcription factor (TF) proteins recognize a small number of DNA sequences with high specificity and control the expression of neighbouring genes. The evolution of TF binding preference has been the subject of a number of recent studies, in which generalized binding profiles have been introduced and used to improve the prediction of new target sites. Generalized profiles are generated by aligning and merging the individual profiles of related TFs. However, the distance metrics and alignment algorithms used to compare the binding profiles have not yet been fully explored or optimized. As a result, binding profiles depend on TF structural information and sometimes may ignore important distinctions between subfamilies. Prediction of the identity or the structural class of a protein that binds to a given DNA pattern will enhance the analysis of microarray and ChIP–chip data where frequently multiple putative targets of usually unknown TFs are predicted. Various comparison metrics and alignment algorithms are evaluated (a total of 105 combinations). We find that local alignments are generally better than global alignments at detecting eukaryotic DNA motif similarities, especially when combined with the sum of squared distances or Pearson’s correlation coefficient comparison metrics. In addition, multiple-alignment strategies for binding profiles and tree-building methods are tested for their efficiency in constructing generalized binding models. A new method for automatic determination of the optimal number of clusters is developed and applied in the construction of a new set of familial binding profiles which improves upon TF classification accuracy. A software tool, STAMP, is developed to host all tested methods and make them publicly available. This work provides a high quality reference set of familial binding profiles and the first comprehensive platform for analysis of DNA profiles. Detecting similarities between DNA motifs is a key step in the comparative study of transcriptional regulation, and the work presented here will form the basis for tool and method development for future transcriptional modeling studies.

Introduction

Transcription factor (TF) proteins usually recognize a small number of DNA targets via the formation of sequence-specific and nonspecific molecular interactions. Understanding the evolution of TF DNA-binding preferences will not only provide useful insights on the mechanism of DNA recognition, it will also allow more accurate prediction of genomic regulatory elements, which still constitutes a major hurdle in understanding cellular gene regulatory networks. Furthermore, high-throughput studies, such as microarray and ChIP–chip, generate a number of DNA motifs that are putative targets of usually unknown TFs. In this study, we present an alignment and comparison platform that is optimized for DNA motifs, thereby allowing for their efficient analysis and enabling and formalizing their evolutionary study. This platform is called STAMP, for similarity, tree-building, and alignment of DNA motifs and profiles.

TF DNA-binding preferences are usually modeled via frequency matrices, derived from alignments of known sites (see Methods). Typically, these position-specific scoring matrices (PSSMs) assume independency between the base positions [1]. It has been recognized that structurally related TFs often share similarities in their DNA-binding motifs, although the extent to which this happens depends on the TF family [2–5]. Generalized binding models or familial binding profiles (FBPs), a term coined by Sandelin and Wasserman [6], constitute an “average” binding specificity of a family of TFs (see Figure 1
Author Summary

Transcription factors are primary regulators of gene expression. They usually recognize short DNA sequences in gene promoters and subsequently alter their transcription rate. It is known that structurally related transcription factors often recognize similar DNA-binding patterns (or motifs). Comparison of these motifs not only provides insights into the evolutionary process they undergo, but it also has many important practical applications. For example, motifs that are found to be “similar” can be combined to form generalized profiles, which can be used to improve our ability to predict novel DNA signals in the promoters of co-expressed genes, and thus facilitate a more accurate mapping of gene-regulatory DNA complexes, together with automatic clustering and classification results, is hoped to shed more light on the evolution of the DNA preferences and their utility in prediction and classification studies.

For more reliable FBP construction methods, and in order to expand the area of applications for generalized binding models, a detailed evaluation of a variety of motif alignment strategies is required. Motif evolution and the motif dependence on the proteins’ structural properties need to be investigated. Sandelin and Wasserman [6] did an important first step when they created a set of 11 FBPs for the nonzinc finger families. During the FBP construction, they noticed that the bZIP family exhibited two different DNA-binding patterns, so they partitioned it into CCAAT/enhancer binding protein (C/EBP)- and cAMP response element-binding (CREB)-related proteins. However, their FBP clustering was done manually and other (sub)family characteristics may have been missed. On the other side of the spectrum, two families may have similar binding preferences, and although they may not belong to the same structural group, it may be reasonable to cluster their binding profiles together, so that the overall number of false positive predictions is reduced. Finally, a detailed analysis of the structural properties of the protein–DNA complexes, together with automatic clustering and classification results, is hoped to shed more light on the evolution of the DNA preferences and their utility in prediction and classification studies.

Schones et al. have compared the effectiveness of three profile distance metrics [13]. We expand their study by evaluating combinations of six distance metrics, three pairwise alignment methods, two multiple-alignment strategies, and two tree-building algorithms. In addition, we develop a new statistic for automatically deciding the optimal number of clusters in a given motif tree. We use this statistic on the tree obtained from the optimal distance metrics and alignment strategies combination to generate a new set of FBPs without prior knowledge of TF structural classification. The new collection of FBPs exhibits better TF classification accuracy than previous manually derived clusters [6] and identifies similarities and differences in the binding preferences of TF (sub)families.

Although species-specific binding preference may emerge for some TFs [15], in general structurally related TFs often share similarities in their DNA-binding preferences. Exploring this trend, Narlikar and Hartemink built a Bayesian TF structural family classifier based on the DNA motifs [10]. We found that the same accuracy of this sophisticated method...
can be achieved with simple motif similarity searches when the appropriate alignment algorithms are used. Correctly predicting the TF structural class for novel motifs will be a crucial step in the interpretation of experiments that aim to systematically estimate regulatory motifs in entire mammalian genomes (e.g., [16]).

Results

Distributions of Similarity Scores in PSSM Columns from Known TFs

All columns from the known PSSM models in the TRANSFAC database [17] were compared with each other using the six metrics presented in Table 1 (see Methods). Figure 2 shows the great variability in the range and distribution of the scores. Both Pearson’s correlation coefficient (PCC) and average log likelihood ratio (ALLR) have negative expected values, which makes them especially suitable for use in alignment algorithms (although, negative mean values can be obtained from any metric by subtracting an appropriate number). Three of the methods, namely PCC, sum of squared distances (SSD), and p-value of chi-square (pCS), have peaks of very small variance, with PCC having two distinct peaks. Comparison of JASPAR [18] columns gave similar results (unpublished data).

Evaluation of the Similarity Metrics in PSSM Column Comparisons

Each of the six metrics was tested for its ability to discriminate between columns randomly sampled from two distinct distributions: an information content–specific distribution and a background (reference) distribution. For information content, I, Figure 3 plots the percent of the $T_I^+$-sampled columns that were included in the area around $T_I^+$ when a false discovery rate (FDR) of 1% was reached. For lower information content, ALLR and SSD perform best at discriminating columns sampled around $T_I^+$ and $F_{I_0}^-$. The finding that ALLR is a better discriminator than pCS metric may seem to contradict the findings of Schones et al. [13]. However, apart from our sampling size being larger, their evaluation focused on the whole motif level. As we will see later, the advantageous performance of ALLR in column-to-column comparisons does not seem to extend to motif-to-motif comparisons.

Comparing Motif Alignment Strategies: The “Best-Hit” Evaluation

It is difficult to construct an unbiased artificial dataset for the evaluation of motif alignment strategies. However, an indication of performance may be gained from similarity searches of a motif against all motifs in a database. Generally, in databases with good representation, the best match to a given motif is expected to be a motif associated with a member of the same structural class [6]. The “best-hit” approach can be used to assess the relative effectiveness of column-scoring metrics and alignment-method combinations by finding the proportion of motifs that match another member of the same structural class using each strategy.

One hundred and five combinations of similarity metrics, alignment methods, and gap penalty values were tested over two datasets: the JASPAR- and TRANSFAC-derived models (see Methods). The top 15 and bottom 15 performing strategies/combinations are presented in Table 2, and a full table of results is available in Table S1. Smith–Waterman local alignments populate the list of best performing results, indicating that they are generally better than Needleman–Wunsch global alignments in motif alignment applications. The results also suggest that the PCC and SSD metrics are on average more effective than the AKL, pCS, and ALLR metrics (including ALLR_LL) for whole-motif comparison. The best-performing combination is Smith–Waterman local alignment using the SSD metric and gap open $= 1$ (average accuracy 0.811), whereas Smith–Waterman local alignment using the PCC metric also scores highly (seventh-best score; average accuracy 0.805). The first strategy that uses the AKL metric appears at position 28 of the list, and the first strategy using ALLR_LL appears at position 37. Strategies using the standard ALLR metric first appear at position 48, and strategies using the pCS metric first appear at position 60.

Predicting the TF Structural Class from its Binding Preferences

In the study of Narlikar and Hartemink [10], a sparse Bayesian learning algorithm was used to predict the structural class of the TF that binds to a given DNA motif. The test dataset in that study consisted of the six largest motif families in TRANSFAC, and Narlikar and Hartemink’s algorithm was able to correctly predict the TF structural family for 86% of these DNA-binding motifs. By analyzing the same dataset, we found that the best-hit approach (with ungapped Smith–Waterman alignment and the PCC metric) yields practically the same performance (87%). The best-hit searches perform better in predicting the bZIP, C4 zinc

| Table 1. The Six Similarity Metrics Used in This Study for PSSM Column Similarity and Motif Alignments |
| Similarity Metric | Formula |
|-------------------|---------|
| Pearson correlation coefficient (PCC) | $PCC(X, Y) = \frac{1}{t} \sum_{b=1}^{B} (\bar{X}_b - \bar{Y}_b) (\bar{X} - \bar{Y})$ |
| Chi-square (pCS) | $\chi^2(X, Y) = \sum_{b=1}^{B} \sum_{k=1}^{K} \left( \frac{X_{bk} - \bar{X}_b}{\sigma^2(X)} + \frac{Y_{bk} - \bar{Y}_b}{\sigma^2(Y)} \right)$ |
| Average Kullback–Leibler (AKL) | $AKL(X, Y) = 10 - \frac{1}{2} \left( \sum_{b=1}^{B} f_b \log \frac{f_b}{\bar{f}_b} + \sum_{b=1}^{B} f_b \log \frac{f_b}{\bar{f}_b} \right)$ |
| Sum of squared distances (SSD) | $SSD(X, Y) = 2 - \frac{1}{t} \sum_{b=1}^{B} (f_b - \bar{f}_b)^2$ |
| Average log-likelihood ratio (ALLR) | $ALLR(X, Y) = \frac{\sum_{b=1}^{B} n_b \log f_b}{\sum_{b=1}^{B} n_b \log \bar{f}_b} - \frac{\sum_{b=1}^{B} n_b \log \bar{f}_b}{\sum_{b=1}^{B} n_b \log \bar{f}_b}$ |
| ALLR with lower limit (ALLR_LL) | Same as above, but a lower limit of −2 is imposed on the score (see text) |

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finger, and Forkhead families, whereas the Bayesian learning is better in the more diverse (in terms of DNA binding motifs) C2H2 zinc finger, homeodomain, and bHLH families (Table 3). We will later show that the appropriate clustering of DNA motifs can help improve the classification accuracy.

Performance of Motif Tree-Building Methods

Sandelin and Wasserman manually constructed FBPs for those ten non-zinc-finger structural families for which four or more motifs exist in the JASPAR database (71 motifs) [6]. One of the ten families, bZIP, produced two distinct FBPs: one related to C/EBP and one related to the CREB proteins. The set of 71 profiles provides an appropriate dataset for testing tree-building strategies and automatic clustering methods. In this study, an agglomerative (UPGMA) and a divisive (SOTA) strategy were compared (see Methods). The most effective alignment strategy for the non-zinc-finger JASPAR dataset, the ungapped Smith–Waterman alignment, was used with each of the six similarity metrics in conjunction with UPGMA or SOTA. Performance was measured as the average homogeneity of the families at each leaf node on the tree and with respect to the tree growth. For a given node, a performance average homogeneity score of 1 denotes that only one family is represented in the motifs clustered at that node (perfect homogeneity); a score of 0.5 denotes that two equally represented families are clustered in that node; etc. The point at which the average homogeneity of the leaf nodes reaches 1 is the point where motifs have been successfully separated on the basis of structural class (although a class might be split into multiple nodes). As can be seen in Figure 4, UPGMA generally performs better than the neural tree.

Figure 2. Distribution of the Observed Scores of Column-to-Column Comparisons for the Five Main Similarity Metrics

Columns are obtained from the TRANSFAC database [17]. The ALLR_LL distribution is identical to ALLR for every point $\geq -2$ (unpublished data). Comparison of the JASPAR motif columns yielded similar results.
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Figure 3. Performance of the Five Main Similarity Metrics in Discriminating between Columns Sampled from Dirichlet Distributions around Information Content $I$ and a Background Distribution

The plot shows the positive predictive rate for an FDR of 1% as a function of the information content.
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Automatic Construction of Familial Binding Profiles

Estimating the optimal number of data clusters on a tree of binding motifs is of significant interest in classification and familial binding property analysis applications. It is well-known, however, that this is an inherently arbitrary procedure; different criteria on where to “split” the tree will give different estimates of cluster number. A number of statistics have been described that aim to estimate the optimal number of clusters (e.g., [19–21]), usually by seeking an optimal balance between intercluster and intrachuster variability.

We used a subset of the JASPAR motifs to understand how different metrics perform in determining the optimal number of clusters in a tree. For this purpose, only closely related members of the ETS, REL, Forkhead, high mobility group (HMG), and MADS families were used. The statistics we compared were: the Gap statistic of Hastie, Tibshirani, and Walter (HTW) [22], the standard Calinski and Harabasz (CH) [19], and a derivative (CH_{log}) we developed (Table 4). When tested on the tree of the five well-defined families, the standard CH statistic didn’t yield any local maximum number of clusters. We believe this is because this statistic performs well when the number of clusters is small compared with the number of points. Otherwise, the within-cluster difference goes quickly to zero, driving the CH to infinity. The CH_{log} by design, avoids this problem. HTW and CH_{log} both gave the correct optimal number of clusters in the five-family dataset. When tested on the full nonzinc-finger JASPAR dataset (71 motifs) (Figure 5), however, HTW and CH_{log} both gave a reasonable number (compared with 11 of Sandelin and Wasserman). For the most part, the identified clusters of motifs seemed reasonable both in terms of binding motif conservation (manual examination) and in terms of TF subfamily classification. More information is provided in the Discussion section. The suitability of CH_{log} was also tested in trees with two, three, four, and six well-defined clusters.
We test the hypothesis that the 17 automatically generated FBPs are a more accurate representation of motif diversity in the JASPAR set than the 11 manually constructed motifs using leave-one-out cross-validation (LOOCV). In this test, we treat the set of FBPs clusters as static multiple alignments and remove the contribution of each motif from its appropriate FBP in turn. The removed motif is then compared against all regenerated FBPs, and treated as correctly classified if it most closely matches the FBP from which it was withdrawn. LOOCV using Sandelin and Wasserman’s 11 manually defined FBPs results in nine misclassifications, or a classification performance of 62/71 (= 87%) (this performance rate was also reported in [6]). By comparison, in our dataset of 17 automatically defined FBPs, LOOCV resulted in two misclassifications, which, combined with the two unclassifiable singleton clusters, suggests a classification performance of 94% (67/71).

JASPAR also contains three zinc-finger motifs that were not used in the Sandelin and Wasserman FBP construction. One of them, C2H2, includes TF proteins with highly divergent patterns of contacts (see Discussion). The other two, DOF (a C4 zinc-finger family) and GATA, have quite conserved DNA-binding patterns. We repeated the above analysis by including the DOF and GATA motifs in JASPAR (four motifs in each family). Our method determined 18 clusters (including two singletons), and a LOOCV test resulted in 72/79 correct classifications (91%). Sandelin’s and Wasserman’s method on the 13 clusters (the previous 11 and the two zinc fingers) resulted in 60/79 correct classifications in the LOOCV test (76%). Compared with the 71-motif tree (Figure 5), the new tree is identical in 15 of the clusters (Figure 7). The main difference is that the two-member cluster of the heterodimeric bHLH proteins, TAL1-TCF3 and HAND1-TCF3, is now split. The TAL1-TCF3 motif is part of a new cluster with the (previously singleton) FOXL1 and the GATA-1 zinc-finger protein motif. The remaining three GATA proteins formed a new cluster. All four DOF proteins are co-clustered with the IRF proteins.

The STAMP Platform

All described methods have been compiled in a software platform (STAMP) (Mahony S, Benos PV, STAMP: A web tool for exploring DNA-binding motif similarities, unpublished). STAMP is modularly designed to allow any combination of column–column scoring metric, alignment method, tree-building algorithm, and multiple-alignment strategy to be used. Its potential uses range from simple motif database searches to identify the TF that may bind to a particular motif to a full-scale analysis of multiple-aligned genomic regions. In the section below, examples of both these uses are provided. STAMP is publicly accessible from http://www.benoslab.pitt.edu/stamp/.

![Figure 4. Average Homogeneity of Families Represented at Each Tree Node as a Factor of the Growth of the Tree](http://www.benoslab.pitt.edu/stamp/)

**Table 3. Performance of TF Structural Family Classification Based on DNA-Binding Preferences in the Six Largest Motif Families in TRANSFAC**

| Name    | Number of TFs | Accuracy       | Best-Hit (STAMP) | Bayesian Learning |
|---------|---------------|----------------|------------------|-------------------|
| bZIP    | 93            | 0.94           | 0.92             |
| C2H2    | 74            | 0.76           | 0.77             |
| C4      | 52            | 0.98           | 0.91             |
| Homeo   | 50            | 0.82           | 0.85             |
| Forkhead| 49            | 0.90           | 0.83             |
| bHLH    | 37            | 0.81           | 0.88             |
| Average | 0.87          | 0.86           |                  |

Results with the database “best-hit” approach (using STAMP) are compared with an earlier work using Bayesian learning classification [10].

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(from the above set of JASPAR PSSMs) and always yielded the correct answer.

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Six PSSM column similarity metrics were evaluated together with three pairwise alignment methods (two gapped and one ungapped), two multiple-alignment strategies, and two tree-building strategies on motif datasets. The results showed that the Smith–Waterman local alignment algorithm used with the PCC or SSD metrics generally performs better in aligning the currently available PSSM models with models for which the associated TF belongs to the same structural family. We also discovered that the high efficiency of some metrics in column-to-column comparison does not extend to the alignment of whole motifs, which is a surprising and previously overlooked outcome. In the case of the ALLR metric, we believe that this inconsistency is due to the metric’s very negatively skewed scoring distribution (Figure 2). Although such a distribution might be advantageous in distinguishing between PSSM columns with low information content, it also makes motif alignment more difficult, especially when the motifs contain low-scoring regions that can rigorously negate the overall score. The difficulties in using ALLR became more apparent with the Needleman–Wunsch global alignment, as low scores that are frequently observed in the beginning and/or at the end of the alignment could not be adequately subsidized by the positive scores in the alignable areas. The use of the alternative ALLR_LL metric (i.e., ALLR with a lower limit of $C_0^2$) improved the results slightly. However, ALLR is the only metric that takes into consideration the background distribution when it compares two columns, which, in combination with the fact that it distinguishes better between columns of low information content (Figure 3), can be advantageous in identifying the correct PSSM model among closely related models (e.g., those belonging to the same family).

Smith–Waterman local alignments were found to be more effective than Needleman–Wunsch global alignments for DNA motifs. This is expected given the current status of the motif databases. Motifs in existing databases usually result from some automated method that runs on a set of unaligned sequences recorded in these databases. These motifs frequently consist of a “core” area of columns with high information content, surrounded by columns of low(er) information content. On such motifs, local motif alignment methods will tend to perform better than global alignment methods. Structurally, this also makes sense, since binding sites are often recognized by a single structural sequence recognition element (e.g., α-helix) either surrounded by or adjacent to a less-specific element that provides additional binding energy. Interestingly, ungapped algorithms or gapped algorithms with high gap-opening penalties generally performed better with the same metric (Table 2). This is probably due to the fact that the motifs for TF families in JASPAR and TRANSFAC share ungapped regions of sim-
The difference between ungapped/high penalty and gapped with lower-gap penalty algorithms is marginal in the JASPAR and TRANSFAC databases. Gapped alignment methods are expected to be more effective when aligning families of motifs that share common half-sites with variable length spacer regions, like many prokaryotic sites. Differences in Classification Efficiency between TF Structural Families

An unexpected finding was that the current multiclass motif classifiers perform no better than simple best-hit similarity queries against a motif database when appropriate motif alignment methods are used. Interestingly, the two zinc-finger families (C2H2 and C4) are predicted with the worst and the best efficiency, respectively, which reflects their binding geometries. The C4 factors form extensive networks of contacts along the length of an α-helix embedded in a B-DNA major groove. As a result, their target sequences are very conserved and thus their predictions easier. The C2H2 zinc fingers on the other hand contact the DNA helix at an angle using only few amino acid side-chains extending from the end of a less intimately associated helix. This results in less binding dependence upon individual amino acid sequences. Changes in certain “key” amino acid positions can drastically alter the DNA-binding specificity, thus yielding highly variable targets.

bZIP factors bind to DNA as dimers in palindromic targets and they select individual half-sites in the process. The monomers readily dissociate from the dimeric form, binding DNA initially as half-site monomers [23]. This kinetic selection process, along with the intimate association of the recognition helix with the major groove (similar to C4 Zn fingers) likely provides exquisite selectivity. The same selectivity may not be realized for the bHLH proteins. Although these factors also select out half-sites, their monomers, in contrast to bZIPs, have very low dissociation rates and act more like covalently linked DNA-binding domains (similar to C2H2 factors). Also, the angle of interaction between the recognition helix and the major groove is more obtuse for bHLH than for bZIP (23 versus 20 degrees), resulting in less interaction with half-sites (3 bp instead of 4 bp). These differences may explain why a bHLH behaves more like a C2H2 in target heterogeneity.

The relatively poor prediction specificity exhibited for the Homeo HTH domain proteins stems from the binding of these factors to highly divergent targets (usually recognizing mostly an "AT" motif), probably due to their dependence upon partner domains that are either dissociable (like Hox-Pbx, Ubx-Exd, and Mat a1-alpha2) or covalently linked (like Oct, Pax, and Pit). Member families of the monomeric wHTH subtype have strong consensus correlations (like the ETS and Forkhead families with consensus GGAA and TAAACA, respectively). This results in higher prediction efficiency for Forkhead than for the more variable Homeo class. It is this idea of variability, perhaps dependent upon multimerization-related constraints, that may be a useful basis for the distinctions we observe.

Table 4. The Four Statistics Tested on Automatic Clustering of the 71 JASPAR Motifs

| Statistic                        | Formula                                                                 |
|---------------------------------|-------------------------------------------------------------------------|
| Hastie, Tibshirani, Walter (HTW)| \( \text{Gap}_s(g) = E_s(\log(W_g)) - \log(W_g) \)                     |
| Calinski and Harabasz (CH)      | \( CH = \frac{B(g)}{W(g)} \)                                          |
| Log-modified Calinski and Harabasz (CHlog) | \( CH_{\log} = \frac{\log(B(g))}{\log(W(g))} \) |

\( B \) and \( W \) are the between- and within-cluster variability, respectively, \( n \) is the number of data points (matrices), and \( g \) is the number of clusters under consideration.

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Figure 6. The Behaviour of the Calinski and Harabasz– Based Log-Metric (CHlog) for the Tree in Figure 4 as the Number of Clusters (g) Is Varied

The value of \( g = 17 \) produces a global minimum in the value of \( CH_{\log} \).

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Another problem that may impose a limit in the classification efficiency of any method (regardless of the TF family) is the quality of the TFBS alignment and the resulting PSSM models. It is hoped that with the accumulation of new data, this will become less of a problem in the future.

Clustering of DNA Profiles: Automatic FBP Construction

Sandelin and Wasserman [6] had previously built 11 FBPs from 71 non-zinc-finger PSSM models (ten TF families) available in the JASPAR database. Their manual clustering of the PSSM models was based on prior knowledge of the structural class of the corresponding TF. An exception to this general rule was the bZIP family, for which they constructed two FBPs (CREB- and C/EBP-related) after observing very different DNA patterns. The FBP corresponding to each structural class was calculated from a multiple-motif alignment where the contributions from outlying motifs were negatively weighted. The 11 familial binding profiles and the 71 motifs in the training set are available to view from the JASPAR database website (http://mordor.cgb.ki.se/cgi-bin/jaspar2005/jaspar_db.pl). Sandelin and Wasserman’s manual approach is suitable for relatively small sets of binding motifs where the structural class corresponding to each motif is known, and where the representatives from each component structural class bind a set of closely related target motifs. However, in the more general case, where families that bind diverse target motifs are included or where the structural class of certain motifs is unknown, it may be useful to attempt automatic generation of the appropriate familial binding motifs.

We developed a fully automated method for PSSM clustering, based on the combinations of metric, alignment strategies, and tree building examined in this study. The advantages of the automatic clustering are obvious. By remaining ignorant to the prior knowledge of the structural class of each motif, we can find cases where motifs from diverse structural classes are more suitably grouped together, if they have similar binding preferences. Similarly, the automatic approach avoids the temptation of forcing together subfamilies of the same structural class with different binding preferences. Also, “outlier” PSSMs can be easily detected through an automatic clustering and subsequently be excluded from the FBP.

The method we used to determine the optimal number of clusters is similar to the Calinski and Harabasz statistic [19], but the intercluster and intraclass variability is calculated on the log-scale. We found this method to compare favourably with other methods on datasets with a known, well-defined small number of clusters and in the whole dataset. When applied on the 71 JASPAR PSSM models of our dataset, this method yielded 17 clusters, two of which are singletons and another two of which contain a pair of heterologous TFs each (Figure 8). Overall, the automatic clustering method divides the dataset into homogeneous clusters with respect to the structural group of the corresponding TF (note that the clusters are based solely on the binding preferences of the TFs). This agrees with the general notion that structurally similar TFs tend to have similar binding specificities. The MADS domain proteins and the wHTH ETS proteins are two examples of TF families with very conserved DNA-binding preferences. Also, homeobox and nuclear receptor family clusters are homogeneous, although some members of these families can be found in other

Figure 7. The tree resulting from a UPGMA tree construction of 12 JASPAR families (79 motifs total) using the PCC scoring metric and Smith–Waterman (ungapped) alignment method. This tree includes the two zinc-finger families (GATA and DOF).

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STAMP: Comparing cis-Regulatory Motifs
sequences in the minor groove with low selectivity. This is
proteins [27]. Both families prefer to bind to AT-rich
family of TFs [26]. The HMGBs are structurally distinct
AT-hook
sented. The HMGA proteins are members of the
HMGB/SOX/SRY, whereas the HMGN family is not repre-
separated by a long linker and they also bind AT-rich
sequences. The HTHs bind to 5-bp and 6-bp recognition
sequences with an interpositioned 6-bp spacer that interacts
with the linker [28]. This explains the long recognition
sequence revealed in the FBP in this cluster.

When the two zinc-finger families were included in the
analysis, the overall structure of the tree and the clusters
remained the same, pointing to the stability of our multiple
alignment and clustering algorithm. Most of the GATA
proteins formed a new cluster, whereas all DOF proteins
joined the cluster of the two IRF proteins. This is because
the DOF consensus target sequence (AAAG) is part of the IRF
motif (Figure 9). The cross-validation results in the extended
family tree/clustering remained very high (91% compared
with 94% in the smaller tree).

The STAMP Platform

The STAMP platform, introduced in this study, contains all
tested algorithms and can be efficiently used in BLAST-like
searches against a database of PSSM models. Various datasets
(including TRANSFAC and JASPAR motifs) are currently
supported. In the future, it would be useful to incorporate
other similarity metrics, alignment methods, and tree-build-
ing algorithms into the platform in order to allow for further
exploration of optimal methods. Note, however, that it may
not be possible to implement all of the known tree-building
algorithms for motif alignment. Other distance-based meth-
ods (such as neighbour-joining [29]) rely heavily on additivity
of the distance metric, which was not possible to define using
our comparison metrics. Parsimony-based methods [30] rely
on the estimation of substitution rates between sites, which is
also not easily definable for frequency matrices. However, a
substitution matrix has recently been defined for DNA-
binding consensus sequences [31], so application of alter-
native tree-building methods may yet be possible in the DNA-
binding motif domain (albeit not for PSSMs per se).

Figure 9. Similarity between the HMG and Forkhead Motifs
These families are grouped together on the HMFGForkhead Group I
cluster (Figure 8).
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In summary, we expect that the methods and the results described in this study will facilitate the exploration of DNA-binding preference evolution amongst related transcription factors and will have a significant impact in many areas of gene research.

Materials and Methods

PSSM column-scoring metrics. A PSSM model of length \( L \) is composed of a set of \( 4 \times L \) weights (columns). Each column, \( X \), follows a probability distribution, \( p_Y(b)_{b \in \{A,C,G,T\}} \), with the base-probability values reflecting the preference of the TF for the corresponding base in this position. The probability values can be estimated from the observed base counts \( N_T(b,\{A,C,G,T\}) \) of \( b \) at position \( y \) in column \( X \), \( f_X(b) = \frac{N_T(b,\{A,C,G,T\})}{\sum_{b \in \{A,C,G,T\}} N_T(b,\{A,C,G,T\})} \). In practice, \( p_Y(b) \) are estimated from \( N_T(b,\{A,C,G,T\}) + \pi \) plus some pseudocounts to reduce small-sample biases and to avoid zero probability values. The assumption of independence between positions is not entirely accurate, but acts as a useful approximation [32–34].

Comparative distances, but more than 99% of the column-to-column scores in the TRANSFAC database [17] were found to have an AKL of less than 10.0. For an important quality: more weight is placed in the high-probability combinations of columns until only one node (the root), which contains a rough alignment of all input PSSMs, and the node model is generated from this alignment. The root node then produces two identical offspring leaf nodes. During each timestep, the algorithm assigns the PSSMs to their most similar leaf nodes and then allows the node model to be updated in accordance with the current alignment the most similar input PSSM (and taking the most similar pair as the start point of the multiple alignment). Once the initial alignment is built, each PSSM is removed from the alignment in turn and realigned to a profile of the other aligned sequences. Iteration of the realignment continues a fixed number of times. For gapped multiple alignments, gaps are encouraged to open in the lowest probability positions and then produced two identical offspring leaf nodes (progressively adding the gap-open and gap-extend penalties in positions of the alignment that already contain gaps.

Tree-building algorithms. We implemented two similarity-based tree-building algorithms: an agglomerative method (UPGMA [44]) and a divisive method that is based on a self-organizing tree algorithm (SOTA [45]). UPGMA begins by assigning each input PSSM its own leaf node. At each timestep, the two nodes with the maximum average pairwise similarity are joined. The tree is built up through successive combinations of nodes until only one node (the root) remains. SOTA does not contain gaps by progressively weighting the gap-open and gap-extend penalties in aligning groups of short motifs. Optimal alignment is sought in both forward and reverse motif directions.

Multiple-motif alignment. Two multiple-motif alignment strategies are tested. One is a progressive profile alignment strategy, which relies on the preconstruction of an approximate guide tree using UPGMA. The multiple alignment is built up by progressively aligning the nodes on the guide tree in order of decreasing similarity. In this way, each internal node contains a “familial” profile. The alignment at the root node will represent the final multiple alignment. The second implemented strategy is iterative refinement alignment, which aims to combat the problem of local minima, common in the progressive alignment methods due to “frozen” subalignments [43]. Iterative refinement builds a robust multiple alignment by progressively adding to the current alignment the most similar input PSSM (and taking the most similar pair as the start point of the multiple alignment). Once the initial alignment is built, each PSSM is removed from the alignment in turn and realigned to a profile of the other aligned sequences. Iteration of the realignment continues a fixed number of times. For gapped multiple alignments, gaps are encouraged to open in the lowest probability positions and then the alignment is updated by progressively weighting the gap-open and gap-extend penalties in positions of the alignment that already contain gaps.
Calinski and Harabasz (CH) [19], and one derivative of the latter (Table 4). For the given dataset, we found that CHlog performed the best. The CHlog formula is given in Table 4, with B and W representing the between and within cluster sum of squared errors, n is the number of matrices (i.e., 71 for the JASPAR dataset), and g is the current number of clusters in the hierarchy. Error here refers to the negative logarithm of the motif comparison p-value (described above). In contrast to the original Calinski and Harabasz statistic, the B and W are log-transformed to avoid the tendency of the denominator towards zero, which results from the relatively small size of the datasets in which we operate. This logarithm transformation means that the estimate for the optimal number of clusters on the tree (K) is the value of g that minimizes CHlog (in the original Calinski and Harabasz formula, the maximum value of CHlog was sought). The suitability of the above metric to the current application domain was confirmed by evaluating its performance in a dataset with a small number of tightly clustered motif groups (see Results).

Motif datasets and structures. For the purposes of testing the accuracy of various motif-alignment and tree-building strategies, we compiled two datasets that contain the motifs of the families with four or more profiles in JASPAR [18] and TRANSFAC (release 9.3 [17]) databases. JASPAR contained 96 motifs in 13 such families (of which 25 motifs belong to three zinc finger families). TRANSFAC contained 586 motifs in 25 families (of which 160 motifs belong to five zinc-finger families). The edges of the motifs were "trimmed" down to the first position with information content 0.4 or more.

Supporting Information

Table S1. Relative Performance of All Tested Motif Alignment Strategies

Performance is measured as the percent of motifs whose structural class are correctly recovered via the best hit in database searches. The two datasets used in this comparison are taken from JASPAR and TRANSFAC. While accuracy was measured over the complete dataset, the results below report separately the performance for the non-zinc-finger families (non-ZNF; ten families in JASPAR, 20 in TRANSFAC) and the zinc-finger families (ZNF; three in JASPAR, five in TRANSFAC). Average is the weighted average. Overlapping (overlap) and ungapped (ungapped) alignments are specified. Gap extension is equal to half the gap opening.

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References

1. Stormo GD (2000) DNA binding sites: Representation and discovery. Bioinformatics 16: 16–23.
2. Suzuki M, Yagi N (1994) DNA recognition code of transcription factors in eukaryotes. Annu Rev Genet 28: 157–163.
3. Rudolph MJ, Gergen JP (2001) DNA-binding by Ig-fold proteins. Nat Struct Biol 8: 384–386.
4. Pabo CO, Peisach E, Grant RA (2001) Design and selection of novel Cys2His2 zinc finger proteins. Annu Rev Biochem 70: 313–340.
5. Auron PE (2004) DNA sequence-specific transcription factors. In: Lotze MT, Trachman AW, editors. Measuring immunity: Basic science and clinical practice. 1st edition. London: Elsevier. pp. 69–101.
6. Sandelin A, Wasserman WW (2004) Constrained binding site diversity within families of transcription factors enhances pattern discovery in promoter sequences. J Mol Biol 339: 207–215.
7. Mahony S, Golden A, Smith TJ, Benos PV (2005) Improved detection of DNA motifs using a self-organized clustering of familial binding profiles. Bioinformatics 21 (Supplement 1): i283–291.
8. Xing EP, Karp RM (2004) MotifPrototypy: A Bayesian profile model for motif families. Proc Natl Acad Sci U S A 101: 10523–10528.
9. Tompa M, Li N, Bailey TL, Church GM, De Moor B, et al. (2005) Assessing computational tools for the discovery of transcription factor binding sites. Nat Biotechnol 23: 137–144.
10. Reeves R, Beckerbauer L (2001) HMGI/Y proteins: Flexible regulators of transcription and chromatin structure. Biochim Biophys Acta 1519: 13–29.
11. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, et al. (2005) MatInspector and beyond: Promoter analysis based on transcription factor binding sites. Bioinformatics 21: 2933–2942.
12. Schones DE, Zumbo P, Zou M (2006) Similarity of position frequency matrices for transcription factor binding sites. BMC Bioinformatics 6: 237.
13. Cartharius K, Frech K, Grote K, Haltmeier M, et al. (2005) MatInspector and beyond: Promoter analysis based on transcription factor binding sites. Bioinformatics 21: 307–313.
14. Maciasac KD, Gordon DB, Neklutova L, Odom DT, Schreiber J, et al. (2006) A hypothesis-based approach for identifying the binding specificity of regulatory proteins from chromatin immunoprecipitation data. Bioinformatics 22: 423–429.
15. Corcoran DL, Feingold E, Dominick J, Wright M, Harnaha J, et al. (2005) Footer: A quantitative comparative genomics method for efficient recognition of cis-regulatory elements. Genome Res 15: 840–847.
16. Xie X, Lu J, Kulbokas EJ, Golub TR, Mootha V, et al. (2005) Systematic discovery of regulatory motifs in human promoters and 3′ UTRs by comparison of several mammals. Nature 434: 338–345.
17. Matsy V, Fricke E, Geffers R, Gossling E, Haubrock M, et al. (2005) TRANSFAC: Transcriptional regulation, from patterns to profiles. Nucleic Acids Res 33: 374–378.
18. Sandelin A, Alkema W, Engstrom P, Wasserman WW, Lenhard B (2004) JASPAR: An open-access database for eukaryotic transcription factor binding profiles. Nucleic Acids Res 32: D91–D94.
19. Calinski T, Harabasz J (1974) A dendrite method for cluster analysis. Communications Statistics 3: 1–27.
20. Davies DL, Bouldin DW (1979) A cluster separation measure. IEEE Trans Patt Anal Mach Intell 1: 224–227.
21. Tibshirani R, Walther G, Hastie T (2001) Estimating the number of data clusters via the Gap statistic. J Roy Stat Soc B 63: 411–423.
22. Hastie T, Tibshirani R, Waleter G (2001) Estimating the number of data clusters via the Gap statistic. J Roy Stat Soc B 63: 411–423.
23. Koller JJ, Metallo SJ, Schneider TL, Schepratz A (1999) DNA specificity enhanced by sequential binding of protein monomers. Proc Natl Acad Sci U S A 96: 11735–11739.
24. Moustakas A, Heldin CH (2005) The nuts and bolts of IRF structure. Nat Struct Biol 10: 874–876.
25. Escalante CR, Brass AL, Pongubala M, Shatova E, Shen L, et al. (2002) Crystal structure of PU.1IRF-4DNA ternary complex. Mol Cell 10: 1097–1105.
26. Reeves R, Beckerbauer L (2001) HMGI/Y proteins: Flexible regulators of transcription and chromatin structure. Biochem Biophys Acta 1519: 13–29.
27. Stott R, Tang GS, Lee KB, Thomas JO (2006) Structure of a complex of tandem HMG boxes and DNA. J Mol Biol 360: 90–104.
28. Xu HE, Rouald MA, Xu W, Epstein JA, Maas RL, et al. (1999) Crystal structure of the human Pax6 paired domain–DNA complex reveals specific roles for the linker region and carboxy-terminal subdomain in DNA binding. Genes Dev 13: 1260–1275.
29. Saitou N, Nei M (1987) The neighbor-joining method: A new method for constructing phylogenetic trees. Mol Biol Evol 4: 406–426.
30. Fitch WM (1971) Toward defining the course of evolution: Minimum change for a specified tree topology. Syst Zool 20: 406–416.
31. Wang T, Stormo GD (2005) Identifying the conserved network of cis-regulatory sites of a eukaryotic genome. Proc Natl Acad Sci U S A 102: 17400–17405.
32. Benos PV, Bulyk ML, Stormo GD (2002) Additivity in protein–DNA interactions: How good an approximation is it? Nucleic Acids Res 30: 17400–17405.
33. Benos PV, Lapedes AS, Fields DS, Stormo GD (2001) SAMIE: Statistical algorithm for modeling interaction energies. PLoS Comput Biol: 115–126.
34. Benos PV, Lapedes AS, Stormo GD (2002) Probabilistic code for DNA recognition by proteins of the EGR family. J Mol Biol 323: 701–727.
35. Hughes JD, Estep PW, Tavazoie S, Church GM (2000) Computational identification of cis-regulatory elements associated with groups of functionally related genes in *Saccharomyces cerevisiae*. J Mol Biol 296: 1295–1314.
36. Pietrokovski S (1996) Searching databases of conserved sequence regions by aligning protein multiple-alignments. Nucleic Acids Res 24: 3850–3855.
37. Aerts S, Van Loo P, Thijs G, Moreau Y, De Moor B (2003) Computational detection of cis-regulatory modules. Bioinformatics 19 (Supplement 2): II5–II14.
38. Roepcke S, Grossmann S, Rahmann S, Vingron M (2005) T-Reg Comparator: An analysis tool for the comparison of position weight matrices. Nucleic Acids Res 33: W438–W441.
39. Wang T, Stormo GD (2003) Combining phylogenetic data with co-regulated genes to identify regulatory motifs. Bioinformatics 19: 2369–2380.
40. Gotoh O (1982) An improved algorithm for matching biological sequences. J Mol Biol 162: 705–708.
41. Needleman SB, Wunsch CD (1970) A general method applicable to the search for similarities in the amino acid sequence of two proteins. J Mol Biol 48: 443–453.
42. Smith TF, Waterman MS (1981) Identification of common molecular subsequences. J Mol Biol 147: 195–197.
43. Barton GJ, Sternberg MJ (1987) A strategy for the rapid multiple alignment of protein sequences. Confidence levels from tertiary structure comparisons. J Mol Biol 198: 327–337.
44. Sokal RR, Michener CD (1958) A statistical method for evaluating systematic relationships. U Kansas Scientific Bull 28: 1409–1438.
45. Dopazo J, Carazo JM (1997) Phylogenetic reconstruction using an unsupervised growing neural network that adapts the topology of a phylogenetic tree. J Mol Evol 44: 226–235.