Evidence for Deep Regulatory Similarities in Early Developmental Programs across Highly Diverged Insects

Majid Kazemian¹,²,†, Kushal Suryamohan³,⁴,†, Jia-Yu Chen¹,†, Yinan Zhang¹, Md. Abul Hassan Samee¹, Marc S. Halfon³,⁴,⁵,⁶,* and Saurabh Sinha¹,⁷,*

¹Department of Computer Science, University of Illinois at Urbana-Champaign
²Laboratory of Molecular Immunology, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland
³Department of Biochemistry, University at Buffalo-State University of New York
⁴NY State Center of Excellence in Bioinformatics and Life Sciences, Buffalo, New York
⁵Department of Biological Sciences, University at Buffalo-State University of New York
⁶Molecular and Cellular Biology Department and Program in Cancer Genetics, Roswell Park Cancer Institute, Buffalo, New York
⁷Institute of Genomic Biology, University of Illinois at Urbana-Champaign

*Corresponding author: E-mail: mshalfon@buffalo.edu; sinhas@illinois.edu.
†These authors contributed equally to this work.

Accepted: August 17, 2014

Abstract

Many genes familiar from Drosophila development, such as the so-called gap, pair-rule, and segment polarity genes, play important roles in the development of other insects and in many cases appear to be deployed in a similar fashion, despite the fact that Drosophila-like “long germband” development is highly derived and confined to a subset of insect families. Whether or not these similarities extend to the regulatory level is unknown. Identification of regulatory regions beyond the well-studied Drosophila has been challenging as even within the Diptera (flies, including mosquitoes) regulatory sequences have diverged past the point of recognition by standard alignment methods. Here, we demonstrate that methods we previously developed for computational cis-regulatory module (CRM) discovery in Drosophila can be used effectively in highly diverged (250–350 Myr) insect species including Anopheles gambiae, Tribolium castaneum, Apis mellifera, and Nasonia vitripennis. In Drosophila, we have successfully used small sets of known CRMs as “training data” to guide the search for other CRMs with related function. We show here that although species-specific CRM training data do not exist, training sets from Drosophila can facilitate CRM discovery in diverged insects. We validate in vivo over a dozen new CRMs, roughly doubling the number of known CRMs in the four non-Drosophila species. Given the growing wealth of Drosophila CRM annotation, these results suggest that extensive regulatory sequence annotation will be possible in newly sequenced insects without recourse to costly and labor-intensive genome-scale experiments. We develop a new method, Regulus, which computes a probabilistic score of similarity based on binding site composition (despite the absence of nucleotide-level sequence alignment), and demonstrate similarity between functionally related CRMs from orthologous loci. Our work represents an important step toward being able to trace the evolutionary history of gene regulatory networks and defining the mechanisms underlying insect evolution.

Key words: cross species enhancer discovery, regulatory modules in diverged insects, alignment free enhancer prediction.

Introduction

The early embryonic development of Drosophila melanogaster (D. mel; “fruit fly”) has been the subject of intensive study, resulting in a sophisticated, albeit incomplete, understanding of the genes involved in pattern formation and extensive characterization of the transcriptional cis-regulatory modules (CRMs) that regulate them. A growing number of studies in divergent insect species, including the Hymenopterans Apis mellifera (A. mel; honeybee) and Nasonia vitripennis (N. vit; jewel wasp), and the Coleopteran Tribolium castaneum (T. cas; red flour beetle), have began to build on this knowledge to explore how developmental gene regulatory networks have evolved over the course of insect diversification. These studies have revealed both striking similarities and
significant differences in the mechanisms of early development among these species (Peel 2008; Rosenberg et al. 2009; Lynch et al. 2012). Intriguingly, many of the observed similarities and differences seem to track less well with phylogeny than with mode of embryonic development. Thus A. mel and N. vit, which as members of the Hymenoptera belong to the most basal clade of the holometabolous insects (fig. 1), appear to be more similar to Drosophila in terms of gap and pair-rule gene expression than does the more closely related beetle T. cas. It has been suggested (Peel 2008) that this is due to the fact that both A. mel and N. vit develop, like Drosophila, using a long-germband mode of embryogenesis (in essence, with most or all segments having their fates established simultaneously prior to gastrulation), whereas T. cas undergoes short-germband development, with progressive determination of segmental fates. This has raised the possibility that the long-germband mode, which is not found outside of the Holometabola, evolved independently in the hymenopteran and dipteran lineages (Peel 2008). In this case, similarities in gap and pair-rule gene expression and regulation might represent convergent evolution, with regulatory relationships and CRMs of comparable function arising de novo and perhaps aided by co-option of transcription factors (TFs) from a different ancestral regulatory program (Heffer and Pick 2013) (fig. 2b). Alternatively, developmental gene expression and regulation in each of these insects could derive by direct descent from the ancestral genome (fig. 2a), with turnover of TF binding sites in orthologous CRM sequences and changes in transacting TFs in the individual lineages leading to the observed differences in their developmental genetics (Lynch, Brent, et al. 2006; Williams et al. 2008).

A key component to understanding regulatory network evolution is analysis of the CRMs that integrate the activities of the orthologous developmental genes. For instance, establishing that orthologous CRMs regulate orthologous genes at multiple steps during development would support the direct descent model, whereas showing use of newly acquired CRMs, or CRMs with distinct binding site compositions and TF combinations, would favor the convergent evolution model. The limited examples of developmental CRMs available today, scattered across different studies, generally point to a scenario of ancestrally derived regulation (Erives and Levine 2004; Zinzen et al. 2006; Goltsev et al. 2007; Cande, Chopra, et al. 2009; Cande, Goltsev, et al. 2009). However, they do not allow us to systematically determine the extent of regulatory network conservation at the level of cis-regulatory mechanism.

Unfortunately, identifying related—by either convergent or direct evolution—CRMs for such a systematic study has presented a considerable challenge. Even among species of the same order, noncoding sequences have diverged beyond the point where standard alignment algorithms are effective, making it impossible to identify homologous noncoding sequences through sequence comparison alone. Computational methods based on clustered TF binding sites (motifs) have proven effective in a small number of cases, primarily from studies of dorsal–ventral patterning (Erives and Levine 2004; Zinzen et al. 2006; Goltsev et al. 2007; Cande, Goltsev, et al. 2009). However, in general such methods have a high false-positive rate, and sufficient knowledge of TFs and motifs is rarely present outside of a few well-studied biological processes (Haeussler and Joly 2011).

Our previous work (Kantorovitz et al. 2009; Kazemian et al. 2011) offers a practical solution to this CRM discovery problem: it does not rely on TFs or their binding motifs, but instead exploits the observation that for many regulatory systems of interest, a small set of known CRMs can be used as training data to facilitate a more complete characterization of related CRMs. We have previously shown (Kantorovitz et al. 2009; Kazemian et al. 2011) that when “training CRMs” from a particular regulatory network are available, this approach is superior to or as effective as existing motif-based methods (Frith et al. 2003) and other methods that use alignment-free scores of sequence similarity (Arunachalam et al. 2010). However, the requirement for training data represents a serious shortcoming. Although extensive data for over 1,850 CRMs, complete with sequences and associated gene
expression patterns, are available for \textit{D. mel} (Gallo et al. 2011), such information is less easily accessible for other model organisms and virtually nonexistent for emerging-model and nonmodel species. For instance, our search of the literature revealed fewer than 20 well-defined CRMs collectively for the three species \textit{An. gam}, \textit{T. cas}, and \textit{A. mel}, and none for \textit{N. vit} (see supplementary note S1, Supplementary Material online). We wondered whether this problem could be overcome by using the wealth of available \textit{Drosophila} data to conduct cross-species CRM discovery in other, diverged insect species. That this would be feasible was by no means certain a priori: the assumptions we had previously tested about sequence similarity of functionally similar CRMs within \textit{Drosophila} would now be put to the test across species separated by up to 350 Myr of insect evolution. (It is worth noting, moreover, that insects, perhaps due to their shorter generation times, have been shown to have a faster rate of genome evolution than vertebrates. For example, flies and honeybees, with their 350-Myr divergence, have only \sim 10\% of orthologous genes in synteny compared with the 50\% seen with the 450 Myr human–pufferfish divergence [Zdobnov and Bork 2007].)

We show here that \textit{Drosophila} training data indeed can enable effective CRM discovery in these species despite their great divergence. We predict over 7,100 new CRMs in four non-Drosophilid insect species and validate 12 of 16 of these as functional in vivo by transgenic reporter gene assays in \textit{Drosophila}. Many of these drive gene expression in a pattern strikingly similar to a known \textit{Drosophila} CRM from the orthologous locus, strongly suggesting that we successfully identified functionally related CRMs despite sequence divergence to the point where the sequences cannot be aligned. Further support for this conclusion comes from analysis of the motif composition of the newly discovered CRMs, which show
substantial similarity in motif content and in some cases, motif organization, when compared with CRMs in the orthologous loci in *Drosophila* (again despite the absence of alignable blocks of conserved sequence). Thus, we find that similar gene expression in highly diverged species is often regulated through similarly constituted CRMs. Our work also suggests that regulatory sequence annotation of diverse insects is an attainable goal without requiring extensive new genome-scale experimental data for each newly sequenced species, and provides a valuable starting point for future attempts to reconstruct the evolutionary history of developmental regulatory networks spanning over 350 Myr of evolution.

**Materials and Methods**

**Statistical Scores**

Each statistical model presented below scores a target sequence window \( S \) for similarity, in terms of \( k \)-mer composition, to a training set of known CRMs from *Drosophila* (see supplementary file S1, Supplementary Material online), denoted by \( C \). These scores are described in detail in Kantorovitz et al. (2009) and Kazemian et al. (2011), and we only outline their main features here. The source codes of the methods are provided in supplementary file S2, Supplementary Material online.

**msHexMCD**

A fifth-order Markov chain is trained on the set of sequences in the training set \( C \) as well as their orthologs from 11 other *Drosophila* genomes. In particular, let \( N_w \) denote the count of 6-mer \( w \) in sequences in \( C \) and their orthologs. This count is first smoothed based on counts \( N_w \) of every 6-mer \( w \) that is one mismatch away from \( w \). Thus, the smoothed count of \( w \) is given by \( \bar{N}_w = N_w + 0.25 \times \sum_{w'} N_{w'} \). Smoothed counts \( \bar{N}_w \) of all 6-mers \( w \) are used to train a fifth-order Markov chain \( \bar{M}^+ \). A different fifth-order Markov chain \( \bar{M}^- \) is trained on a set of background sequences, also taken from *Drosophila*. The score of sequence \( S \) is the log-likelihood ratio (LLR) of the two Markov chains on \( S \), that is, \( \text{msHexMCD}(S) = \log(\text{Pr}[S|\bar{M}^+] / \text{Pr}[S|\bar{M}^-]) \).

**msIMM**

We trained a fifth-order interpolated Markov model (IMM*) (introduced by Salzberg et al. [1998] and re-implemented in Kazemian et al. [2011]) on the set of sequences in the training set \( C \) as well as their orthologs from 11 other *Drosophila* genomes. An IMM combines all Markov chains of up to certain order (here 5). It provides a natural extension to a fixed order Markov chain and can better capture the natural variability of TF binding sites (see Kazemian et al. 2011 for more details). An IMM (IMM*) is trained separately on a set of background sequences. The score of sequence \( S \) is the LLR of the two IMM* on \( S \), that is, \( \text{msIMM}(S) = \log(\text{Pr}[S|\text{IMM*}] / \text{Pr}[S|\text{IMM*^-}]) \).

**PAC-rc**

This score quantifies the overrepresentation of every 6-mer \( w \in W \) in sequences in \( C \), relative to a set of background sequences, assuming a Poisson distribution of word counts. The score is based on van Helden (2004) and reimplemented in Kantorovitz et al. (2009). Let \( n_w \) and \( \lambda_w \) denote the count of \( w \) in \( C \) and a size-matched set of background sequences, respectively. The score of sequence \( S \) is defined as: \( \text{PAC-rc}(S) = \frac{1}{|W|} \sum_{w \in W} F(\lambda_w, n_w - 1) \), where \( F(\lambda, n) \) is the Poisson cumulative distribution function (CDF) with mean \( \lambda \).

**Global Assessment Using Expression Gene Sets: The “Evaluation P Value”**

As an initial assessment, we statistically examined whether the predicted CRMs of a regulatory system in a non-*Drosophila* (target) genome are located near the genes related to that system. Here, a regulatory system refers to an expression domain or stage, the genes expressed in that domain or stage (expression gene set), and the CRMs that drive expression in the domain or stage. For each regulatory system and each non-*Drosophila* target genome, we first inferred the expression gene sets \( G \) from relevant expression gene sets in *Drosophila* using homology maps downloaded from the Inparanoid database (Ostlund et al. 2010). For each target genome, we also created a gene universe \( U \) as all genes with a homolog in *Drosophila* according to the Inparanoid database. The expression gene sets and universes are provided in supplementary file S1, Supplementary Material online. Given a collection of predicted CRMs, we collected the nearest neighboring gene for each of the top scoring windows until this set of genes, \( P \), is of size 200 genes. We finally examined the statistical significance using a Hypergeometric test \( H(n, P, G, U) \), where \( n \) is the number of genes in both \( P \) and \( G \). We call this the evaluation \( P \) value. (We used a fixed number of genes in set \( P \) rather than genes defined by a score threshold, in order to ensure that \( P \) values from different Hypergeometric tests are based on the same sample size. The size of 200 genes was chosen following Kantorovitz et al. [2009].)

**Drosophila Reporter Constructs and Transgenic Analysis**

Genomic sequences were generated by polymerase chain reaction using genomic DNA from the appropriate species, cloned into pJet1.2blunt (Fermentas), and confirmed by sequencing. Primer sequences are provided in supplementary table S8, Supplementary Material online. The putative CRM sequences were subcloned into plasmid pattBnucGFPs, a φC31-enabled *Drosophila* transformation vector containing Enhanced Green Fluorescent Protein (EGFP) under the control of a minimal hsp70 promoter (details available on request), using XbaI + Xhol. Transgenic flies were produced by Genetic Services Inc. (Cambridge, MA) by injection into lines attP2 or attP40. Homozygous transgenic embryos were collected, fixed, and stained using standard methods.
Regulus

(Complete details in supplementary file S3, Supplementary Material online.) Here, we compute a probabilistic score of similarity between two sequences \(x \) and \( y \) in terms of their binding site composition. This score allows for the uncertainty of binding site prediction and for the varying relevance of different types of binding sites. It is a likelihood ratio of a “homology model” denoted by “\( M \)”, and a null model denoted by “\( nb \).” In the null model, the sequences \( x \) and \( y \) are independently generated by a zeroth-order hidden Markov model (HMM) similar to Sinha et al. (2003). The HMM has one state for each input motif \( m \) and one background state \( bg \). The HMM stochastically enters a state, stochastically emits a single nucleotide (if the state is \( bg \)) or a single site (if in a motif state), and repeats this process until it has emitted a nucleotide sequence of the predetermined length, that is, length of \( x \) or \( y \). Transition probabilities for each state are independent of the current state and depend only on the state being entered. These transition probabilities are learned by maximum-likelihood estimation from an appropriate set of training CRMs and reflect the density of motif matches in those CRMs. After estimation, we adjust the motif transition probabilities by scaling by a constant so that the average of all motif transition probabilities is 0.001. (Details at the end of this section.) Emission probabilities in the background state are determined by nucleotide frequencies in the given sequences, and those in a motif state are determined by the PWM form of the motif. Let \( T \) represents the sequence of (hidden) states entered by the HMM in generating the two sequences \( S = \{ x, y \} \), then \( Pr(T|HMM) \) denotes the product of all transition probabilities corresponding to \( T \), and \( Pr(S|T) \) is the probability of emitting the given sequences conditional on \( T \). The likelihood of \( S \) under the null model \( nb \) is then given by

\[
Pr(S|nb) = \sum_T Pr(T|HMM)Pr(S|T)
\]

with the sum being taken over all possibilities of the hidden state path \( T \) generating both sequences.

The homology model is very similar to the above, with the exception that the probability of a state path \( T \), that is, \( Pr(T) \) above, is now scaled by a multiplicative factor \( \varphi(T) \) that reflects the similarity of motif composition of the two sequences, according to \( T \). In particular, if \( T \) includes \( n_m^x \) visits to the motif state \( m \) in generating sequence \( x \) and \( n_m^y \) visits to the same motif state in generating sequence \( y \), that is, if \( T \) prescribes \( n_m^x \) sites of \( m \) in \( x \) and \( n_m^y \) sites of \( m \) in \( y \), then

\[
\varphi(T) = \varphi^{\min(n_m^x,n_m^y)},
\]

where \( \varphi \) is a constant (set to 5 by default). Thus, the similarity score induced by the stochastic (and hidden) state path \( T \) is an exponential function of the shared numbers of sites of each motif, summed over all motifs. Now, the probability of state...
path \( T \) under the homology model \( s \) defined as being proportional to \( \varphi(T)\Pr(T|\text{HMM}) \), that is

\[
\Pr(T|M) = \frac{\varphi(T)\Pr(T|\text{HMM})}{\sum_T \varphi(T)\Pr(T|\text{HMM})}.
\]

Finally, the likelihood of \( S \) under the homology model \( M \) is given by

\[
\Pr(S|M) = \sum_T \Pr(T|M)\Pr(S|T).
\]

The Regulus score for the given pair of sequence \( S = \{x,y\} \) is the LLR given as follows:

\[
\text{LLR} = \log \frac{\Pr(S|M)}{\Pr(S|\text{HMM})}.
\]

Everything else being equal if one pair of sequences has more shared sites for a motif \( m \), that is, a greater value of \( \min(n_{\text{ms}}, n_{\text{ms}}') \), the LLR score will be greater (supplementary file S3, Supplementary Material online). Note that the null model \( \text{HMM} \) assumes the same HMM, rather than two separate HMMs, have generated the pair of sequences. This corresponds to the null hypothesis that the two sequences independently mirror some aspects of the binding site composition of training CRMs, and thus provides a strong baseline to contrast with some aspects of the binding site composition of training null hypothesis that the two sequences independently mirror the same state for each motif) mentioned above. Thus, if there are \( k \) motifs, the sum of motif transition probabilities in the final HMM is \( k \times 0.001 \), and the remaining probability mass is assigned to the background state.

**Results**

**Motifs Used in Regulus**

For A/P patterning CRMs we used motifs for the TFs BCD, CAD, GT, HB, KNI, KR, HKB, TLL, PKH, and CIC and for D/V patterning CRMs we used motifs for TFs DL, BRK, SNA, VND, IND, CIC, TWI, ZEN, and ESPL. All motifs were obtained from FlyFactorSurvey (Zhu et al. 2011).

**Transition Probabilities in Regulus**

For each motif, a motif density was estimated by training a two-state HMM (one state representing motif and one state for background), using the Baum–Welch algorithm, on an appropriate set of sequences. We used the training set of CRMs associated with A/P patterning and D/V patterning (“ap” and “dv,” respectively, supplementary file S1, Supplementary Material online) in this step. The motif densities were then scaled so as to have an average of 0.001, to obtain transition probabilities in the combined HMM (with one state for each motif) mentioned above. Thus, if there

**BLAST Analysis and Dot Plot Alignments**

Intergenic sequences flanking each developmental gene were extended up to 20 kb, terminating at the beginning of the next coding region. Sequence pairs of \( D. \) mel plus another species were then analyzed for sequence similarity using the NCBI (National Center for Biotechnology Information) BLAST server (Boratyn et al. 2013) with parameters customized for improved cross-species comparison (-W 9, -G 1, -E 2, -r 1, -q -1; see Korf et al. 2003). For each \( D. \) mel sequence, BLAST was performed 1) against the orthologous \( D. \) mojavensis or \( D. \) virilis locus (for twi, \( D. \) willistoni); 2) against the orthologous locus from one or more of \( A. \) gam, \( T. \) cas, \( N. \) vit and \( A. \) mel; and 3) against 1,000 randomized sequences obtained by shuffling the respective non-\( D. \) mel sequence. For even skipped alignments, sequence from \( T. \) cas, \( A. \) mel and \( D. \) mojavensis was also analyzed. To gauge the distribution of the bit scores of the resulting BLAST High Scoring Pairs (HSPs), box plots (showing median, inter-quartile range (IQR), 1.5 x IQR) of the top 50 BLAST hits for each of the BLAST runs were generated and statistical significance was tested by performing pairwise comparisons using Tukey’s multiple comparison test (see supplementary table S9, Supplementary Material online, for contingency matrix of \( P \) values).

Dot plot alignments were generated using the EMBoss dottup program (Rice et al. 2000) with window size = 9 and step size = 1.

Intergenic sequences flanking each developmental gene were extended up to 20 kb, terminating at the beginning of the next coding region. Sequence pairs of \( D. \) mel plus another species were then analyzed for sequence similarity using the NCBI (National Center for Biotechnology Information) BLAST server (Boratyn et al. 2013) with parameters customized for improved cross-species comparison (-W 9, -G 1, -E 2, -r 1, -q -1; see Korf et al. 2003). For each \( D. \) mel sequence, BLAST was performed 1) against the orthologous \( D. \) mojavensis or \( D. \) virilis locus (for twi, \( D. \) willistoni); 2) against the orthologous locus from one or more of \( A. \) gam, \( T. \) cas, \( N. \) vit and \( A. \) mel; and 3) against 1,000 randomized sequences obtained by shuffling the respective non-\( D. \) mel sequence. For even skipped alignments, sequence from \( T. \) cas, \( A. \) mel and \( D. \) mojavensis was also analyzed. To gauge the distribution of the bit scores of the resulting BLAST High Scoring Pairs (HSPs), box plots (showing median, inter-quartile range (IQR), 1.5 x IQR) of the top 50 BLAST hits for each of the BLAST runs were generated and statistical significance was tested by performing pairwise comparisons using Tukey’s multiple comparison test (see supplementary table S9, Supplementary Material online, for contingency matrix of \( P \) values).

Dot plot alignments were generated using the EMBoss dottup program (Rice et al. 2000) with window size = 9 and step size = 1.
et al. 2008; Schetelig et al. 2008; Schinko et al. 2008; Choe and Brown 2009; Keller et al. 2010; Lynch and Desplan 2010; Wilson et al. 2010; Wilson and Dearden 2011, 2012; Duncan et al. 2013), the segment polarity genes vg and engrailed (Nie et al. 2001; Lynch, Brent, et al. 2006; Choe and Brown 2009; Duncan et al. 2013), Sox family members (Wilson and Dearden 2008), and others (Papatsenko et al. 2011). Thus, it is reasonable to hypothesize that regulatory relationships and CRM sequences may also be similar.

We next sought to determine whether orthologous CRMs could be identified through alignment of noncoding regions surrounding developmental genes of interest. Previous results indicated that even within the Diptera, flies and mosquitoes have diverged too much for most noncoding sequences to align (Engstrom et al. 2007; Sieglaff et al. 2009). We used a sensitive BLAST analysis along with analysis of dot-matrix alignments to look for regions of local alignment in the intergenic regions flanking developmentally important genes with similar expression in Drosophila and the other four species. We were unable to align any of the sequences we tested (fig. 3 and supplementary fig. S1, Supplementary Material online). Thus, if CRMs have been conserved, it has been at a level undetectable by sequence alignment. In order to identify the relevant CRMs, therefore, we adapted a method we previously developed for finding functionally related CRMs within the Drosophila genome for cross-species CRM discovery.

Cross-Species Supervised CRM Prediction Pipeline

Our basic approach to CRM prediction (fig. 4a) is to use a training set of Drosophila CRMs, defined by a common functional characterization (e.g., mesoderm expression), to build a statistical model that captures the short subsequence ($k$-mer count distribution in these CRMs. An appropriate set of background sequences (e.g., random segments of the noncoding genome) is also used in this training phase. Note that CRMs in the training set regulate related, but often not identical gene expression (fig. 4a, top). The trained model is then used to score every 500-bp long window in the “target genome,” and the highest peaks in the resulting score profile are predicted to be CRMs. This “supervised CRM prediction” approach was demonstrated in our previous work (Kantorovitz et al. 2009; Kazemian et al. 2011) to accurately predict CRMs for many different regulatory systems in D. mel, using respective training sets from the same species (available in supplementary file S1, Supplementary Material online). We extended this approach to predict CRMs in the four other insect genomes—An. gam, T. cas, A. mel, and N. vit—by using the trained models from Drosophila CRMs to scan each of these target genomes separately. These genomes are greatly diverged from D. mel with estimates of last common ancestors ranging from 250 Ma (An. gam) to 350 Ma (A. mel) (Wiegmann et al. 2009, 2011). We hypothesized that despite our inability to align noncoding sequences between these genomes, the underlying cis-regulatory similarity would be strong enough that the $k$-mer composition of a CRM in the target genome would be significantly similar to that of Drosophila CRMs active in similar spatiotemporal domains.

As a preliminary assessment of the viability of our cross-species approach, we tested statistically whether the candidate CRMs in each target genome are enriched near genes with expression patterns (inferred from homology to Drosophila) matching those conferred by the training CRMs. Such enrichment would be expected if the candidate CRMs are functionally similar to the training set (fig. 4a, “Evaluation”). This was indeed the case, with evaluation $P$ values $\leq$ 1E-5 (Hypergeometric test, see Materials and Methods) observed for 11–19 of 36 different regulatory systems in each species (fig. 4b and supplementary table S2, Supplementary Material online), and with 13 data sets exhibiting significant $P$ values in at least three non-Drosophila genomes. The three methods exhibit complementary strengths, as noted also in Kantorovitz et al. (2009) and Kazemian et al. (2011), with several cases where $P$ values on the same data set differ substantially between methods (supplementary fig. S2, Supplementary Material online). We refer to a data set with evaluation $P$ value $\leq$ 1E-5 as “amenable” to prediction under its respective statistical model. Note that although the evaluation $P$ values reveal general trends such as which species or which regulatory systems are more amenable to the approach, they do not suggest expected error rates for prediction. They are also likely to underestimate statistical significance both because gene expression patterns in the target genomes were inferred based on homology to D. mel genes and because the $P$ values are calculated based on the assumption that candidate CRMs regulate their nearest neighboring genes. However, complete gene expression data are often unavailable both for D. mel and for the other species, and the correct regulated genes will not always be the closest ones.

It is worth noting that our motif-blind, $k$-mer-based approach has been previously demonstrated as being superior to or comparable to more popular, motif-based methods for CRM discovery in D. mel (Kantorovitz et al. 2009; Kazemian et al. 2011). Here, we performed a similar comparison on non-Drosophila species for the two data sets (anterior–posterior and dorsoventral patterning) where relevant TFs and motifs are best known. Performance of motif-based methods on
FIG. 3.—Alignment of intergenic regions of developmental genes between diverged species. Examples in this figure are from the even skipped (eve) locus; see supplementary figure S1, Supplementary Material online, for examples from other loci. (a) Dotplot alignment of Drosophila mojavensis and D. melanogaster (~60 Ma) downstream intergenic regions shows clear alignment (diagonal) between the two species. (b) The more diverged Sepsid fly Themira putris (~75 Ma) lies near the edge of noncoding alignment (weak diagonal) as previously reported by Crocker and Erives (2008) and Hare et al. (2008). Neither mosquitoes (c) nor beetles (d) show recognizable alignment, as can be seen from the absence of a visible diagonal and similarity to alignment with randomized sequence (e). The dotplot results are confirmed by BLAST analysis (f), which shows that only D. mojavensis and T. putris have a BLAST score distribution with scores exceeding those obtained from randomized sequence (for D. mel–T. putris vs. D. mel–randomized T. putris, $P < 0.024$, uncorrected one-sided Student’s t-test).
FIG. 4.—(a) Pipeline for cross-species supervised CRM prediction. Top: A set of CRMs that regulate similar gene expression patterns is selected as a training set. Expression driven by CRMs from the blastoderm (left) and CNS (right) training sets are pictured. Note that there is a range of related but nonidentical patterns. (Blastoderm embryo pictures are adapted from Schroeder et al. [2004] under the terms of the CC-BY license.) A statistical model is then trained on k-mers in this training set of CRMs as well as non-CRM sequences from Drosophila. Separately, an “expression gene set” is defined as Drosophila genes with expression patterns matching the training set. Middle: The trained model is used to scan a non-Drosophila target genome, and score every 500-bp window in the genome for similarity to the training set in Drosophila. Highest scoring windows (marked with asterisks) are predicted to be CRMs. Bottom: The expression gene set in Drosophila is mapped through homology to a gene set (in the target genome) whose expression is expected to be similar to that of predicted CRMs. Genes near predicted CRMs are tested for enrichment in this gene set, providing a preliminary statistical assessment (evaluation $P$ value) of the predictions. *Additional data sets amenable to supervised CRM prediction in Drosophila are shown in supplementary table S2, Supplementary Material online. (b) Data sets amenable to cross-species supervised CRM prediction. Shown are the data sets where an evaluation $P$ value was calculated.

| Training CRM set (CRMs) | Drosophila | Anopheles | Apsi | Nazania | Tribolium |
|--------------------------|------------|-----------|------|---------|-----------|
| blastoderm (94)          |            |           |      |         |           |
| ev (55)                  |            |           |      |         |           |
| wing (9)                 |            |           |      |         |           |
| CNS (66)                 |            |           |      |         |           |
| imaginal disc (81)       |            |           |      |         |           |
| eye (29)                 |            |           |      |         |           |
| ectoderm (74)            |            |           |      |         |           |
| imaginal disc (14)       |            |           |      |         |           |
| ventral ectoderm (18)    |            |           |      |         |           |
| en ederm (36)            |            |           |      |         |           |
| ev_neurogenet ectoderm (8)|          |           |      |         |           |
| ectoderm (54)            |            |           |      |         |           |
| sors ectoderm (14)       |            |           |      |         |           |
| neuroectoderm (9)        |            |           |      |         |           |
| brane (65)               |            |           |      |         |           |
| somatic, muscle (40)     |            |           |      |         |           |
| salivary gland (14)      |            |           |      |         |           |
| # amenable data sets     | 25*         | 11         | 19   | 12      | 16        |

(a) Pipeline for cross-species supervised CRM prediction. Top: A set of CRMs that regulate similar gene expression patterns is selected as a training set. Expression driven by CRMs from the blastoderm (left) and CNS (right) training sets are pictured. Note that there is a range of related but nonidentical patterns. (Blastoderm embryo pictures are adapted from Schroeder et al. [2004] under the terms of the CC-BY license.) A statistical model is then trained on k-mers in this training set of CRMs as well as non-CRM sequences from Drosophila. Separately, an “expression gene set” is defined as Drosophila genes with expression patterns matching the training set. Middle: The trained model is used to scan a non-Drosophila target genome, and score every 500-bp window in the genome for similarity to the training set in Drosophila. Highest scoring windows (marked with asterisks) are predicted to be CRMs. Bottom: The expression gene set in Drosophila is mapped through homology to a gene set (in the target genome) whose expression is expected to be similar to that of predicted CRMs. Genes near predicted CRMs are tested for enrichment in this gene set, providing a preliminary statistical assessment (evaluation $P$ value) of the predictions. *Additional data sets amenable to supervised CRM prediction in Drosophila are shown in supplementary table S2, Supplementary Material online. (b) Data sets amenable to cross-species supervised CRM prediction. Shown are the data sets where an evaluation $P$ value was calculated.
both data sets was poor for all non-\textit{Drosophila} target species (supplementary table S3, Supplementary Material online), further supporting our choice of \textit{k}-mer-based methods for cross-species CRM discovery.

\textbf{Effective Recovery of Known CRMs in Non-\textit{Drosophila} Species}

To facilitate validation of our predictions, we focused our attention on those candidate CRMs that were located near genes with an expression pattern (either known or inferred by orthology to \textit{D. mel}) that matches the training set. As discussed above, although not all orthologs are expressed in related patterns, most of the patterning genes that have been studied have similar expression to their \textit{D. mel} orthologs during at least some developmental stages. We reasoned that restricting our search to the neighborhood of such genes would simplify interpretation of any observed expression because we would accordingly have a reasonable expectation of the expression pattern associated with a candidate CRM. We therefore redefined a candidate CRM, corresponding to a particular statistical model, as one with the following properties: 1) The data set being used is amenable to prediction, 2) the candidate CRM is located within 20 kbp of a gene whose \textit{Drosophila} ortholog is annotated with an expression pattern matching the training set, 3) its score (global rank) is in the top 0.5\% of all segments in the target genome, and 4) it is one of the highest scoring segments within 50 kbp to either side of the gene (“local rank” \( \leq 2 \)). Approximately 7,100 sequences met these criteria (supplementary table S4, Supplementary Material online). To evaluate this scheme, especially the newly introduced “local rank” criterion, we examined how a set of 16 experimentally verified CRMs in non-\textit{Drosophila} insect genomes, known from the literature, scored with our models (fig. 4c). Remarkably, 12 of the 16 bona fide CRMs were ranked at 1 or 2 of approximately 400–500 scored segments in their respective locus (of length 108 kbp on average) by at least one of the models. Once again we noted complementarity among the three models, with each model placing the known CRM in the top two local ranks in 5–9 of the 16 instances. In 7 of the 12 cases with high local rank, the known CRM did not meet the stringent global rank criterion used in our previous work on the \textit{Drosophila} genome (Kantorovitz et al. 2009; Kazemian et al. 2011); however, the global rank was always better than the weaker 0.5\% threshold introduced above, justifying our choice of using both local and global ranks to define candidate CRMs (supplementary table S5, Supplementary Material online). The extremely limited number of verified CRMs in the species of interest precludes a more comprehensive in silico evaluation that includes specificity estimates.

\textbf{In Vivo Validation of Candidate CRMs}

We selected a set of 24 segments, of length 500–1,000 bp, from among the four insect species to test for functional activity through in vivo reporter gene assays (supplementary table S6, Supplementary Material online). Due to the relative difficulty (\textit{T. cas}, \textit{An. gam}) or current inability (\textit{A. mel}, \textit{N. vit}) to make transgenic animals using native hosts, sequences were cloned from their native species and tested in transgenic \textit{Drosophila}. (We note that the few examples of CRMs reported in the literature for these other species similarly have been tested through reporter assays in \textit{Drosophila}.) Roughly two-thirds of these test segments (15/24) were chosen from the candidate CRMs (as defined above); the remainder were chosen to allow assessment of false positives and did not score sufficiently high in terms of global and/or local rank. Overall, the 24 test segments were selected to span a broad range of global ranks (4–60,512 of \~1 million windows; median = 528) and local ranks (1–50 of \~430 windows per gene; median = 1) under the three prediction models from ten different training sets. Moreover, the sequences not qualifying as candidate CRMs were chosen from loci for which we were also testing high-scoring candidates. This served as a control for the possibility that any sequence selected from the locus would function as a gene-specific CRM. A detailed characterization of the test set is provided in supplementary table S6, Supplementary Material online.

The experimental assays described above confirmed reporter gene activity for 16 CRMs in four non-\textit{Drosophila} insect genomes, nearly doubling the number currently described (fig. 4a and supplementary fig. S3 and table S6, Supplementary Material online). Of these, 12 (75\%) were among the sequences “predicted” by one of the models and are thus considered to be true positive results; the remaining four are considered false negative results (see \( 2 \times 2 \) confusion matrix in fig. S5b). Three of the 15 candidate CRMs failed to drive gene expression in our reporter assay and are considered to be false positive results. Thus, in the selected set of 24

\begin{figure}[h]
\centering
\caption{Continued}
\end{figure}
segments, we observed a precision of 80% and a recall of 75%, with an F1 score of 77%. The accuracy of predicting negatives was lower, with specificity equaling 63%. Requiring a pre-

This is significant, as each training set contains a range of similar, but not identical, expression patterns. Coincidental activity due to similarity in k-mer profile between a candidate CRM and the training set would therefore not be expected to precisely match a specifically chosen gene’s expression. In con-

contrast to the true positives, only one of the four false negatives, that is, test segments that drove expression but did not meet the criteria for candidate CRMs, clearly matched the expression of the associated gene. The remaining three may partially overlap, but not fully encompass, regulatory sequences (fig. 5a, J and C, and supplementary fig. S3, Supplementary Material online). Furthermore, for 8 of the 12 true positive predictions (75%), the reporter gene expression pattern we observed fits at least one high-scoring training set (fig. 5c; if a segment meets the criteria of a candidate CRM as per a given model, supplementary table S6, Supplementary Material online).
bryos reminiscent of two known (Ochoa-Espinosa et al. 2005) (Nvit_h_m8; stripes 1 and 5) (Fig. 5). The GEMSTAT model predicts similar expression profiles for both CRMs (overlapping the endogenous endogenous stripe 5 and extends almost to the posterior margin of stripe 6 ("Nvit_h_m8", rightmost green bar). The GEMSTAT model predicts similar expression profiles for both CRMs (Dmel_h_stripe_2+6-GEMSTAT, Nvit_h_m8-GEMSTAT), with a posterior stripe overlapping the endogenous hairy stripe 6 and a broad anterior domain that straddles endogenous D. mel stripes 1 and 2. (b) Modeled gene expression regulated by sog CRMs in D. mel ("Dmel_sog_shadow"; Hong et al. 2008) and An. gam ("An. gam_sog_1," this study). The endogenous expression pattern of sog in D. mel is ectodermal (blue) and agrees with the GEMSTAT-predicted profile (red) for the Dmel_sog_shadow CRM. The same model predicts a mesodermal expression pattern as output of the Agam_sog_1 CRM as reported in this work.

The endogenous expression pattern of sog in D. mel and An. gam is ectodermal (blue) and agrees with the GEMSTAT-predicted profile (red) for the Dmel_sog_shadow CRM. The same model predicts a mesodermal expression pattern as output of the Agam_sog_1 CRM as reported in this work.

Highly Related Expression Patterns Suggest Identification of Homologous CRM Pairs

In several cases, the reporter gene expression not only matches the expression pattern of the putative associated gene but also closely resembles the activity of a known Drosophila CRM from the orthologous locus. For instance, the activity of a T. cas wg CRM is like that of the wg_Avg enhancer (Von Ohlen and Hooper 1997) (Tcas_wg_1; fig. 5A), whereas a T. cas Dichaete CRM recapitulates postblastoderm expression driven by the D.Difth_O-E enhancer (Ochoa-Espinosa et al. 2005) (Tcas_D_1; figs. 5A.C and 4A.D). Similarly, an N. vit CRM located in the N. vit hairy locus drives gene expression in two stripes in Drosophila embryos reminiscent of two known D. mel CRMs: h_stripe_6+2 (ET15 in Howard and Struhl [1990]) and h_ET5 (expressing in h stripes 1 and 5) (Nvit_h_m8; fig. 5A.E; Howard and Struhl 1990). A comparison between the N. vit CRM and the Drosophila h_stripe_6+2 CRM indicates an overall similarity of binding site composition (supplementary fig. S4A, Supplementary Material online). To further explore this similarity, we used a quantitative model (GEMSTAT for A/P CRMs, see Materials and Methods) to predict the expression driven by the two sequences. Encouragingly, the model predicts highly similar expression readout from the two sequences, with a pattern that closely matches that observed for the N. vit CRM (fig. 6A). Thus, despite a complete lack of nucleotide-level alignment (see below), these beetle and wasp CRMs appear to be homologous to their Drosophila CRM counterparts: they are drawn from orthologous loci, have related binding site composition (see below), and share common regulatory activity when tested in the same transregulatory context.

A CRM from An. gam sog Maintains the Mosquito, Rather Than the Fly, Expression Pattern

Of particular note is a CRM discovered in the An. gam sog locus. Drosophila sog is expressed in the neurogenic ectoderm and mesectoderm, resolving primarily to the mesectoderm by mid germband extension. In contrast, the An. gam sog CRM drives reporter gene expression in the mesoderm (An. gam_sog_1; fig. 5J). This pattern is consistent with the described expression of An. gam sog, which is mesodermally, not ectodermally, expressed, perhaps due to utilization of a different combination of cis-regulatory inputs, or of the same inputs with different strengths, by the responsible CRM (Golslev et al. 2007). This is supported by our GEMSTAT modeling, which predicts ectodermal and mesodermal expression domains for the D. mel and An. gam CRMs, respectively (fig. 6B). Importantly, the candidate CRM sequence from An. gam retains An. gam sog regulatory activity even when placed into the D. mel genome. This provides one of the relatively few known examples of regulatory evolution at the cis-level that has led to qualitative (Jeong et al. 2008) rather than quantitative (Wunderlich et al. 2012) expression divergence.

CRM Pairs Exhibit Significant Similarity at the TF Binding Site Level, but Not at the Sequence Level

We showed above that noncoding regions are not alignable between Drosophila and the other studied species for a subset of orthologous loci (fig. 2 and supplementary fig. S1,
Supplementary Material online). To ensure that our newly identified CRMs did not represent exceptions to this, we used LASTZ (Harris 2007) to test for conserved segments between each newly verified non-Drosophila CRM and a 20-kbp region on either side (plus introns) of the orthologous Drosophila target gene. The results, consistent with our findings in figure 2 and supplementary figure S1, Supplementary Material online, show that alignment between related pairs of CRMs is not distinguishable from that between unrelated pairs of sequences (fig. 7; also see supplementary

![Diagram](image-url)
online, and data not shown), we could in many cases identify the complete lack of alignable noncoding sequence between motif makeup within each pair of CRMs. Moreover, despite the results from Regulus, we observed similarity in overall patterns of motif distribution into better relief. Consistent with well-conserved motifs and bring potentially important patterns with the anterior–posterior or dorsal–ventral patterning systems, for which several relevant motifs are known. The first measure casts each sequence as a vector of motif scores and compares two vectors using the cosine similarity measure (see supplementary note S3, Supplementary Material online, titled “MoCS”). This score was unable to discriminate related sequence pairs.

The second measure, called Regulus, is a probabilistic score that reflects the number of binding sites common to both sequences, while properly accounting for variable strengths of a TF’s binding sites and variable frequencies of different TFs’ sites (see Materials and Methods). To our knowledge, there is no obvious comparator in the literature for Regulus as a similarity score of two CRMs based on their motif compositions. (The obvious approach of counting every motif’s occurrence in either CRM and comparing these count vectors was explored in the MoCS score mentioned above, and its variants that are not reported here.) The Regulus score was able to map the non-Drosophila CRMs to the correct Drosophila gene locus with an accuracy of 50% (11 of 22 CRMs mapped correctly, Binomial test P value = 4 \times 10^{-5}, fig. 7b), compared with the 18% accuracy (4 of 22, P value = 0.34) on the same set of CRMs when using LASTZ as the similarity measure.

To help us better interpret the Regulus results, and to further explore the binding site content of the CRMs, we made a graphic map of the identified motifs along the sequences for several pairs of CRMs (from this study as well as from the literature; fig. 8 and supplementary fig. S4a–e, Supplementary Material online). Because multiple species of flies, bees, and mosquitoes have now been sequenced, we included orthologous regions (based on alignment within a given subfamily or genus) of the CRMs for each of the respective D. mel, An. gam, and A. mel CRMs where possible. These multispecies alignments help to focus attention on the most well-conserved motifs and bring potentially important patterns of motif distribution into better relief. Consistent with the results from Regulus, we observed similarity in overall motif makeup within each pair of CRMs. Moreover, despite the complete lack of alignable noncoding sequence between genera (supplementary fig. S1, Supplementary Material online, and data not shown), we could in many cases identify a rough “core” region with a loose linear correlation of motifs. This was particularly apparent between the more closely related flies and mosquitoes for the early embryonic twi and sog CRMs, which share regions of heavily interspersed DORSAL and ZELDA motifs (fig. 8), and the brinker (brk) CRMs, with DORSAL/SNAIL/ZELDA regions (supplementary fig. S4b, Supplementary Material online). We observed a striking correspondence in motif pattern between our newly discovered An. gam sog CRM (Agam_sog_1), the An. gam sog CRM previously described by Cande, Goltsev, et al. (2009), and the D. mel CRMs sog_broad_lateral_neurogenic_ectoderm and sog_NEEsog_shadow (fig. 8b and data not shown). Similarly, a clear motif consensus could be seen among the CRMs driving early expression of sim in flies, mosquitoes, and bees, with the CRMs from all three families having a core of DORSAL, ZELDA, and SNAIL sites (supplementary fig. S4c, Supplementary Material online). Although a clearly defined core pattern could not be defined for comparisons with the N. vit h and T. cas D CRMs, an overall resemblance of motif composition and arrangement is still observed (supplementary fig. S4a and d, Supplementary Material online).

Taken together, our motif-level analysis, along with our observations on the functionally homologous pairs of CRMs above (for sog, wg, h, and D), strongly indicates that CRMs may diverge well beyond recognition by nucleotide-level alignment methods while still maintaining their overall cis-regulatory logic, at least in part through similar motif composition.

Discussion

Are the Candidate CRMs True CRMs?

Our candidate CRM sequences were selected due to k-mer profile similarity to known Drosophila CRMs. A trivial interpretation of the results, therefore, would be that because the candidates have a fly-like k-mer profile, they drive fly-like reporter gene expression without being true regulatory elements in their native species. Although virtually all currently identified insect CRMs were defined through validation by reporter gene assays in transgenic Drosophila (e.g., Wolff et al. 1998; Erives and Levine 2004; Markstein et al. 2004; Zinzen et al. 2006; Goltsev et al. 2007; Cande, Chopra, et al. 2009; Cande, Goltsev, et al. 2009; Ayyar et al. 2010), and Drosophila reporter gene assays have even been used to demonstrate deep regulatory homologies between fly and mouse CRMs (Awgulewitsch and Jacobs 1992; Malicki et al. 1992; Xu et al. 1999), the only definitive way to rule out this possibility would be to conduct reporter gene assays in each individual species (which is not currently possible for A. mel and N. vit). Nevertheless, there is considerable evidence to support the view that the identified candidates represent true CRMs in their respective species. In particular, a substantial number of the CRMs drove reporter gene expression not only in the generally expected pattern (e.g., blastoderm, peripheral...
FIG. 8.—TF binding site motifs in functionally related CRM pairs. Binding site motifs are indicated by vertical colored bars, with bar heights correlating to degree of match to the motif. Horizontal green bars indicate the extent of the sequences tested in vivo. Dashed lines indicate motif similarities among nonorthologous CRMs or CRMs from different genera. For clarity, only a subset of conserved motifs are marked. (a) Motif alignment of the D. mel “twi_dl_mel” twi CRM and the orthologous sequence from four other Drosophilids with the An. gam twi CRM from Cande, Goltsev, et al. (2009). Blue bars highlight the similar “core” motif arrangement of sites for DORSAL lying between sites for ZELDA. Note the conserved arrangement of motifs as evidenced by lack of crossing of the dashed lines. (b) sog CRMs from Drosophila and Anopheles. The An. gam sog CRM from Cande, Goltsev, et al. (2009) is shown in the middle, aligned with two orthologous Anopheles sequences. The entire pictured sequence was confirmed in vivo. Aligned sequences from the Drosophila sog_broad_lateral_ectodermal_enhancer (BLNEE) are shown at the bottom. Alignments at the top represent the An. gam sog_1 CRM (this study, fig. 5I) from An. gam and An. epiroticus. Close motif alignment can be observed between all three sets of CRMs, and for the CRM from Cande, Goltsev, et al. (2009) and its Drosophila counterparts in particular. Species abbreviations: Agam, Anopheles gambiae; Aara, An. arabiensis; Aepi, An. epiroticus; Aqua, An. quadriannulatus; Amer, An. merus; Amelas, An. melas; Dmel, Drosophila melanogaster; Dsec, D. sechellia; Dyak, D. yakuba; Dpse, D. pseudoobscura; Dana, D. ananassae; Dwil, D. willistoni; Dvir, D. virilis.
nervous system) but also in the specific pattern expected for the associated gene. Indeed, in several cases the CRM-driven expression closely resembled that regulated by known Drosophila CRMs from the orthologous locus. Although it is conceivable that sequences coincidentally matching the training set’s k-mer profile may exist and drive generally related gene expression, note that each training set encompasses a range of nonidentical expression patterns. For instance, the blastoderm training set includes genes expressed at different positions within the blastoderm embryo, genes expressed in either of the A/P or D/V axes, and both gap-like and pair-rule-like patterns. Similarly, the CNS data set includes genes expressed in the brain, the lateral portion of the ventral nerve cord, and the midline of the ventral nerve cord. Given this diversity in expression pattern even within a training set, we expect that although a coincidental “CRM-like” sequence might drive expression that resembles some aspects of the training set patterns, it is highly unlikely—especially given the observed lack of sequence conservation—that such sequences would be maintained in an orthologous locus, drive the expression pattern expected for the orthologous gene, and recapitulate the regulatory activity of a specific D. mel CRM associated with that gene. Thus, the Nvit_h_1 CRM drives a two-stripe h-like pattern rather than, say, a broad posterior gap gene pattern. Furthermore, the An. gam sog CRM we identified drove gene expression not in the D. mel sog pattern, but in the An. gam sog pattern similar to expression from other known An. gam sog CRMs. This strongly suggests that the sequence represents a true An. gam sog CRM. Collectively, these data provide a compelling argument in favor of the candidate CRMs being bona fide regulatory elements in their own species, and not merely emulating Drosophila CRMs by virtue of coincidental sequence similarities.

Cross-Species CRM Prediction

One goal of this work was to determine whether Drosophila data could be used to predict CRMs in highly diverged species, on a genomic scale; previous cross-species predictions have only been performed on a locus-by-locus basis (e.g., Cande, Goltsev, et al. 2009). In our previous work (Kantorovitz et al. 2009; Kazemian et al. 2011), we reported on extensive comparisons among different approaches to within-species supervised CRM prediction in Drosophila. We also evaluated the complementary strategy of identifying TF motifs overrepresented in the training CRMs, using a collection of known motifs for Drosophila, followed by motif-based CRM prediction using HMM models (Frith et al. 2003; Sinha et al. 2003). The lesson from these evaluations was that our three scores for supervised CRM prediction—msHexMCD, mslMM, and PAC-rc—were equivalent or superior to any other methods available at the time, including more popular methods based on motif matches. (We reconfirmed the advantage over motif-based methods in this work.) Hence, using these three scores for cross-species prediction was a natural choice, and we leave the assessment of other k-mer-based statistical scores (Leung and Eisen 2009; Lee et al. 2011; Ren et al. 2013) on this new problem as future work. We also note that the potential of k-mer-based scores in cross-species CRM characterization was illustrated by Peterson et al. (2009), who used aggregates of short word matches to assign orthology relationships to pairs of validated CRMs in the eve locus between D. melanogaster and tephritid (true fruit fly) genomes.

Cross-species prediction presents special challenges for evaluating results, as the expression pattern of CRM target genes in the native organism is frequently not known, and even known expression patterns cannot always be mapped cleanly between different organisms. In choosing candidates for validation here, we attempted to focus on genes whose expression patterns were expected either to be reasonably well-conserved, or previously described. Although alterations in gene expression pattern between species complicate evaluation, we expect that our methodology will nevertheless prove robust to such changes, as evidenced by our successful prediction of the An. gam sog mesodermal CRM. Altered expression due to changes in the distribution of transacting factors should of course have no effect on our sequence-based supervised prediction. However, as our method scores the statistical distribution of k-mers and not direct counts or identification of specific binding sites, it should also be able to predict CRMs which drive an altered pattern of gene expression due to gain or loss of a specific TF but which integrate an overall similar set of regulatory inputs.

Direct Homology versus Convergent Evolution

Previous studies of dorsal–ventral patterning in non-Drosophilid insects point to weakly conserved regulatory mechanisms with a high degree of turnover and exchange of binding sites (Erives and Levine 2004; Zinzen et al. 2006; Goltsev et al. 2007; Cande, Goltsev, et al. 2009). A small number of identified CRMs regulating later embryonic development of the heart also support the idea of conserved regulatory mechanisms and homologous CRMs (Cande, Chopra, et al. 2009). Very few data exist with respect to patterning in the anterior–posterior axis. CRMs have been identified for T. cas hunchback (hb) and hairy (h) (Wolff et al. 1998; Eckert et al. 2004), but although both drive appropriate expression in Drosophila embryos, suggesting that the basic cis–trans regulatory mechanisms have been conserved, neither has been characterized at the binding site level. A 4-kb noncoding fragment from the A. mel gt locus containing binding sites for upstream factors such as Cad, Hb, and Otd drives expression in Drosophila in a pattern consistent with A. mel gt expression (Wilson and Dearden 2011), but the large size of the fragment
makes it difficult to draw definitive conclusions about CRM composition.

Our newly identified CRMs, like many of the previously known non-Drosophila CRMs, reside in similar positions relative to their associated genes as do their Drosophila counterparts, and we have shown for several of these CRM pairs—in particular, for sog and twi—strikingly similar TF binding site motif composition and arrangement. (The presence of shared binding sites in short blocks of sequence conservation between otherwise diverged CRM sequences was also noted in Hare et al. [2008].) Overall, this suggests a mechanism of direct evolutionary descent with various degrees of binding site turnover, gain or loss of additional binding sites, and/or changes in expression of TFs accounting for species-specific differences in regulatory activity. These mechanisms have been observed before (e.g., Williams et al. 2008), but generally not over evolutionary distances as vast as those considered here. Taher et al. (2011) were able to identify a number of putatively directly related CRMs between distant species by performing “conservation tunneling” in which they were able to detect alignments between each species and an intermediate species even in the absence of alignable noncoding sequence between the two more distant species (i.e., human–frog, frog–zebrafish alignments to detect human–zebrafish-related CRMs). However, whole-genome sequences of appropriately positioned insect species to allow such an approach are not currently available, and the rapid radiation of the Insecta makes it questionable whether such intermediate species could be found. Moreover, as noted earlier, the fly–honeybee sequence divergence appears to be more extensive than the human–fish divergence, despite the shorter calendar years (vs. generations) of separation (Zdobnov and Bork 2007).

Nevertheless, given the lack of alignable sequences between the homologous pairs of CRMs, we cannot rule out convergent evolutionary mechanisms. Convergent evolution has been implicated in a number of examples, such as the de novo emergence of the Pomc neuronal CRMs in the mammalian lineage (Domene et al. 2013) and the identification of several cross-functional urochordate and vertebrate regulatory sequences by Doglio et al. (2013). The CRM compositions we observe could also represent a mix of direct descent and convergent changes, as has been suggested for CRMs for the shavenbaby gene in several Drosophila lineages (Frankel et al. 2012). Similarity of motif compositions between functionally similar CRMs, while presumed above as evidence of shared descent, may also result from parallel evolution, if for instance the “solution space” of a specific regulatory functionality is limited and necessitates a unique combination of binding sites (Crocker and Eries 2008). Additional complications may arise when considering “shadow enhancers,” that is, functionally redundant CRMs for the same gene (Hong et al. 2008), believed to confer robustness of expression readouts (Frankel et al. 2010). It is possible for such redundant pairs (or sets) of CRMs to arise by convergent evolution, for example, the Pomc neuronal CRMs mentioned above (Franchini et al. 2011), or to exhibit differences in binding site compositions, for example, the sog NEE and LSE enhancers (Markstein et al. 2002). This in turn complicates assessing divergent versus convergent evolution in functionally similar CRMs from two species. Much more extensive CRM discovery covering a fuller range of intermediate species and a more comprehensive coverage of each locus will be needed to begin addressing these questions—something the approach we introduce here will be able to facilitate. For a more elaborate discussion of different modes of CRM evolution, we refer the reader to Robinson et al. (2011) and the associated journal issue.

Taken as a whole, the results from our cross-species, motif-blind supervised CRM prediction and motif-based comparison suggest that we have been able to discover genuine CRMs in four diverged insect species, at least some of which share function with their Drosophila counterparts. Importantly, our high rate of success in cross-species supervised CRM prediction suggests that the large and ever-growing amount of available experimental data on Drosophila CRMs can be leveraged effectively to provide regulatory element annotation for the many insect genomes that have been, or are in the process of being, sequenced. Our results argue strongly that despite extensive binding site turnover and overall sequence divergence, similar regulatory mechanisms govern developmental gene expression even over distances of >350 Myr, and suggest that gene regulatory networks have been directly conserved. Our work provides a possible way forward for detection of and reasoning about regulatory DNA homology, analogous to but going beyond the highly successful BLAST-based paradigm for coding sequences. This will involve the powerful combination of alignment-free methods to predict putative homologs in the orthologous gene locus and experimental methods to characterize the expression readout of the predicted homolog. Complementing the computational tools with experimental validation will likely be necessary, especially for large evolutionary distances, to discern functional homology from the superficial similarity of two sequences that share some common inputs (TF binding sites) but distinct outputs. Depending upon the particular species and CRMs of interest additional computational methods may be of assistance, including alignments of either or both of the remote homologs with their respective “close relatives” (such as in fig. 8), quantitative modeling of expression readout from CRM sequence (such as in fig. 6), and motif-based comparisons (such as with Regulus, or similar tools of the future) if possible. Whole-genome chromatin state profiles (Celniker et al. 2009) and massively parallel enhancer assays (Arnold et al. 2013) will also play an important role in such an endeavor. Our work also highlights some of the subtler challenges in this paradigm, such as the problem of defining or detecting homology of CRMs in the face of potentially diverged trans environments;
the An. gambiae sog CRM is a case in point. As the paradigm matures and additional related CRMs from the growing number of sequenced insect genomes become identified, we will be able to trace the evolutionary history of these diverged regulatory networks and determine the various direct and convergent evolutionary influences that shaped the developmental genetics of the vast insect radiation.

**Supplementary Material**

Supplementary files S1–S3, notes S1–S4, figures S1–S4, and tables S1–S9 are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).

**Acknowledgments**

The authors thank Scott Barolo, Sue Brown, Claude Desplan, Thom Kaufman, Gene Robinson, Steve Small, the Developmental Studies Hybridoma Bank, and the MR4 consortium for materials and reagents, Qiyun Zhu for help with reporter constructs, Jack Leatherbarrow and Jocelin Rimes for support for fly genetics, and Michael Buck for comments on the manuscript. They also thank the B10 (ten bee) Genome Project (G.E. Robinson and G. Zhang, principal investigators) for sharing genome sequences from which orthologs of A. mel CRMs were extracted based on alignments. NIH grant 1 R01 HG85233 to M.S.H. and S.S. and USDA grant 2011-04656 to M.S.H. and S.S.

**Literature Cited**

Aranda M, Marques-Souza H, Bayer T, Tautz D. 2008. The role of the segmentation gene hairy in Tribolium. Dev Genes Evol. 218: 465–477.

Arnold CD, et al. 2013. Genome-wide quantitative enhancer activity maps identified by STAR-seq. Science 339:1074–1077.

Arunachalam M, Jayasurya K, Tomancak P, Ohler U. 2010. An alignment-free method to identify candidate orthologous enhancers in multiple Drosophila genomes. Bioinformatics 26:2109–2115.

Auwgulewitsch A, Jacobs D. 1992. Deformed autoregulatory element from Drosophila functions in a conserved manner in transgenic mice. Nature 358:341–344.

Ayyar S, Nagaraju S, Simpson P, Stollewerk A. 2010. An arthropod cis-regulatory element functioning in sensory organ precursor development dates back to the Cambrian. BMC Biol. 8:127.

Bergman CM, Carlson JW, Celniker SE. 2005. Drosophila DNase I footprint database: a systematic genome annotation of transcription factor binding sites in the fruitfly, Drosophila melanogaster. Bioinformatics 21:1747–1749.

Boratyn GM, et al. 2013. BLAST: a more efficient report with usability improvements. Nucleic Acids Res. 41:W29–W33.

Bucher G, Klingler M. 2004. Divergent segmentation mechanism in the Tribolium ortholog of knirps and knirps-related is crucial for head segmentation but plays a minor role during abdominal patterning. Dev Biol. 321: 284–294.

Choe CP, Brown SJ. 2009. Genetic regulation of engrailed and wingless in Tribolium segmentation and the evolution of pair-rule segmentation. Dev Biol. 325:482–491.

Crocker J, Erives A. 2008. A closer look at the eve stripe 2 enhancers of Drosophila and Themis. PLoS Genet. 4:e1000276.

Doglio L, et al. 2013. Parallel evolution of chordate cis-regulatory code for development. PLoS Genet. 9:e1003904.

Domene S, et al. 2013. Enhancer turnover and conserved regulatory function in vertebrate evolution. Philos Trans R Soc Lond B Biol Sci. 368:20130027.

Duncan Ej, Benton MA, Dearden PK. 2013. Canonical terminal patterning is an evolutionary novelty. Dev Biol. 377:245–261.

Eckert C, Aranda M, Wolff C, Tautz D. 2004. Separable stripe enhancer elements for the pair-rule gene hairy in the beetle Tribolium. EMBO Rep. 5:638–642.

Engstrom PG, Ho SJ, Drivenes O, Becker TS, Lenhardt B. 2007. Genomic regulatory blocks underlie extensive microsynteny conservation in insects. Genome Res. 17:1898–1906.

Erives A, Levine M. 2004. Coordinate enhancers share common organizational features in the Drosophila genome. Proc Natl Acad Sci U S A. 101:2851–2856.

Franchini LF, et al. 2011. Convergent evolution of two mammalian neuronal enhancers by sequential exaptation of unrelated retroposons. Proc Natl Acad Sci U S A. 108:15270–15275.

Frankel N, et al. 2010. Phenotypic robustness conferred by apparently redundant transcriptional enhancers. Nature 466:490–493.

Frankel N, Wang S, Stern DL. 2012. Conserved regulatory architecture underlies parallel genetic changes and convergent phenotypic evolution. Proc Natl Acad Sci U S A. 109:20975–20979.

Frith MC, Li MC, Weng Z. 2003. Cluster-Buster: finding dense clusters of motifs in DNA sequences. Nucleic Acids Res. 31: 3666–3668.

Gallo SM, et al. 2011. REDfly v3.0: toward a comprehensive database of transcriptional regulatory elements in Drosophila. Nucleic Acids Res. 39:D118–D123.

Goltsev Y, et al. 2007. Evolution of the dorsal-ventral patterning network in the mosquito, Anopheles gambiae. Development 134: 2415–2424.

Haussler M, Joly JS. 2011. When needles look like hay: how to find tissue-specific enhancers in model organism genomes. Dev Biol. 350: 239–254.

Hare EE, Peterson BK, Iyer VN, Meier R, Eisen MB. 2008. Sepsid even-skipped enhancers are functionally conserved in Drosophila despite lack of sequence conservation. PLoS Genet. 4:e1000106.

Harris RS 2007., Improved pairwise alignment of genomic DNA [PhD thesis]. Department of Computer Science and Engineering, The Pennsylvania State University.

He X, Samee MA, Blatti C, Sinha S. 2010. Thermodynamics-based models of transcriptional regulation by enhancers: the roles of synergistic activation, cooperative binding and short-range repression. PLoS Comput Biol. 6(9):e1000935.

Heffer A, Pick L. 2013. Conservation and variation in Hox genes: how generic interactions within the tinman complex of the flour beetle, Tribolium castaneum. Development 136:3153–3160.

Celniker SE, et al. 2009. Unlocking the secrets of the genome. Nature 459: 927–930.

Cerny AC, Grossmann D, Bucher G, Klingler M. 2008. The Tribolium ortholog of knirps and knirps-related is crucial for head segmentation but plays a minor role during abdominal patterning. Dev Biol. 321: 284–294.

Choe CP, Brown SJ. 2009. Genetic regulation of engrailed and wingless in Tribolium segmentation and the evolution of pair-rule segmentation. Dev Biol. 325:482–491.

Hare EE, Peterson BK, Iyer VN, Meier R, Eisen MB. 2008. Sepsid even-skipped enhancers are functionally conserved in Drosophila despite lack of sequence conservation. PLoS Genet. 4:e1000106.

Harris RS 2007., Improved pairwise alignment of genomic DNA [PhD thesis]. Department of Computer Science and Engineering, The Pennsylvania State University.

He X, Samee MA, Blatti C, Sinha S. 2010. Thermodynamics-based models of transcriptional regulation by enhancers: the roles of synergistic activation, cooperative binding and short-range repression. PLoS Comput Biol. 6(9):e1000935.

Heffer A, Pick L. 2013. Conservation and variation in Hox genes: how generic interactions within the tinman complex of the flour beetle, Tribolium castaneum. Development 136:3153–3160.
Olesnicky EC, et al. 2006. A caudal mRNA gradient controls dorsoventral organization in the honeybee (Apis mellifera). PLoS Biol. 8:120.

Jeong S, et al. 2008. The evolution of gene regulation underlies a morphological difference between two Drosophila sister species. Cell 132: 783–793.

Kantorovitz MR, et al. 2009. Motif-blind, genome-wide discovery of cis-regulatory modules in Drosophila and mouse. Dev Cell. 17: 568–579.

Kazemian M, Zhu Q, Halton MS, Sinha S. 2011. Improved accuracy of supervised CRM discovery with interpolated Markov models and cross-species comparison. Nucleic Acids Res. 39:9463–9472.

Keller RG, Desplan C, Rosenberg M. 2010. Identification and characterization of Nasonia Pax genes. Insect Mol Biol. 19(Suppl 1): 109–120.

Korf I, Yandell M, Bedell J 2003. BLAST, O'Reilly Media, Incorporated.

Kazemian M, Zhu Q, Halfon MS, Sinha S. 2011. Improved accuracy of supervised CRM discovery with interpolated Markov models and cross-species comparison. Nucleic Acids Res. 39:9463–9472.

Keller RG, Desplan C, Rosenberg M. 2010. Identification and characterization of Nasonia Pax genes. Insect Mol Biol. 19(Suppl 1): 109–120.

Korf I, Yandell M, Bedell J 2003. BLAST, O'Reilly Media, Incorporated.

Kazemian M, Zhu Q, Halfon MS, Sinha S. 2011. Improved accuracy of supervised CRM discovery with interpolated Markov models and cross-species comparison. Nucleic Acids Res. 39:9463–9472.

Keller RG, Desplan C, Rosenberg M. 2010. Identification and characterization of Nasonia Pax genes. Insect Mol Biol. 19(Suppl 1): 109–120.

Korf I, Yandell M, Bedell J 2003. BLAST, O'Reilly Media, Incorporated.

Kazemian M, Zhu Q, Halfon MS, Sinha S. 2011. Improved accuracy of supervised CRM discovery with interpolated Markov models and cross-species comparison. Nucleic Acids Res. 39:9463–9472.

Keller RG, Desplan C, Rosenberg M. 2010. Identification and characterization of Nasonia Pax genes. Insect Mol Biol. 19(Suppl 1): 109–120.

Korf I, Yandell M, Bedell J 2003. BLAST, O'Reilly Media, Incorporated.

Kazemian M, Zhu Q, Halfon MS, Sinha S. 2011. Improved accuracy of supervised CRM discovery with interpolated Markov models and cross-species comparison. Nucleic Acids Res. 39:9463–9472.
Wunderlich Z, et al. 2012. Dissecting sources of quantitative gene expression pattern divergence between Drosophila species. Mol Syst Biol. 8: 604.

Xu PX, et al. 1999. Regulation of Pax6 expression is conserved between mice and flies. Development 126:383–395.

Zdobnov EM, Bork P. 2007. Quantification of insect genome divergence. Trends Genet. 23:16–20.

Zhu LJ, et al. 2011. FlyFactorSurvey: a database of Drosophila transcription factor binding specificities determined using the bacterial one-hybrid system. Nucleic Acids Res. 39:D111–D117.

Zinzen RP, Cande J, Ronshaugen M, Papatsenko D, Levine M. 2006. Evolution of the ventral midline in insect embryos. Dev Cell. 11:895–902.

Associate editor: Patricia Wittkopp