Gene expression

Balancing protein similarity and gene co-expression reveals new links between genetic conservation and developmental diversity in invertebrates

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ABSTRACT
Motivation: To identify genetic conservation relative to precise aspects of developmental diversity, an essential question in computational biology, we developed a new comparative method that allows conserved modules for the best balance between protein sequence similarity and gene co-expression to be constructed, in invertebrates.

Results: Our method, referred to as the best-balance constraint procedure (BBCP), yielded 719 functionally conserved modules (FCMs) comprising 2–23 gene pairs. These modules were consistent with the developmental roles of orthologues as inferred from Gene Ontology, RNAi knockouts, InterPro and process-specific microarray data. New relationships were defined between genetic conservation and developmental diversity. Novel gene associations were indeed found in 94% of the FCMs. 150 modules being completely new. A significant proportion of the FCMs (18%, 132 modules) described cell type-specific mechanisms, comprising neuronal, muscle and germ cell signaling, new associations being found in 125 modules. Also found were gene associations for cell fate specification activities previously not highlighted by computational means, e.g., in FCMs containing homologues. These data indicate that highly discriminative description of genetic conservation can be deduced using BBCP, and reveal new correlations between cellular and developmental diversity and gene essentiality in invertebrates.

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Supplementary information: For supplementary information, please refer to Bioinformatics online.

INTRODUCTION
To compare the development of an organism with another, it is important to consider the properties of developmental systems (Rudel and Sommer, 2003) and the biological roles of genes and their encoded products in the context of complex gene regulatory networks (Davidson et al., 2002; Hinman et al., 2003; Rast, 2003). The availability of genome sequences and genome-wide biological attributes provides a large amount of information that can be analyzed in silico in order to detect developmental genetic mechanisms that may be conserved among different organisms. In this respect, the analysis of gene co-expression was applied to the comparison of two or more different organisms (Teichmann and Babu, 2002; Stuart et al., 2003; van Noort et al., 2003; Bergmann et al., 2004; McCarroll et al., 2004). One of these studies reported a global decomposition of conserved expression from six evolutionarily distant organisms, and was based on modules that do not necessarily contain only homologous proteins (Bergmann et al., 2004). The other studies were focused on functional conservation. Two studies compared Saccharomyces cerevisiae and Caenorhabditis elegans, defining orthologous relationships using either the best reciprocal hit approach (Teichmann and Babu, 2002) or phylogenetic trees (van Noort et al., 2003). Stuart et al. (2003) performed a global clustering of genetic conservation in yeast, C.elegans, Drosophila melanogaster and human, using pairs of orthologous proteins as defined by best reciprocal hits. McCarroll et al. (2004) analyzed aging in C.elegans and Drosophila using one-to-one orthologs as generated by combining BLASTP, phylogenetic trees and Smith–Waterman alignments. Considering the best orthologs may, however, not allow conserved modules to be retrieved at high sensitivity for two reasons. First, complex biological processes may involve a strong inter-specific diversity among organisms, which implies that orthologous proteins with rather divergent sequences may be involved in similar biological processes or molecular functions. This is for example the case with Hox genes in regulating morphogenesis (Aboobaker and Blaxter, 2003) or members of the nuclear receptor family in regulating the function of C.elegans lateral hypodermal cells (Miyabayashi et al., 1999). Second, the search for best orthologs may often return a gene that is not the nearest phylogenetic neighbor of the query sequence (Koski and Golding, 2001). Thus, we hypothesized that the computational analysis of conserved biological processes might gain in informativity and accuracy if performed by applying a new method, referred to here as the ‘best-balance constraint procedure’ (BBCP), that takes into account the influence of protein similarity and gene co-expression with no a priori. To test our hypothesis, we applied the BBCP method to the comparison of C.elegans (The C.elegans Sequencing Consortium, 1998; Kim et al., 2001) and Drosophila (Adams et al., 2000; Arbeitman et al., 2002). To test for BBCP validity and discriminating power, we used Gene Ontology (GO) annotations (Ashburner et al., 2000), InterPro domain annotations (Mulder et al., 2003), RNAi phenotypes resulting from genome-wide analyses of C.elegans.

as they describing new aspects of cellular and physiological diversity in *C.elegans* and *Drosophila*.

METHODS

Construction of functionally conserved modules

We developed a new comparative approach, the BBCP method, as described below. Functional conservation may be associated with several constraints during evolution. These constraints may result in a limited number of alternatives to achieve a given biological process, which may be reflected by conservation at different levels, e.g. at the level of gene sequence, gene regulation or pathway activity. The analysis of these phenomena thus requires methods based on the combination of different information. Previous work in this respect combined sequence information with information about pathway topology (Forst and Schulten, 1999, 2001). Other examples comprise the alignment of protein interaction networks across species through the analysis of sequence similarity (Kelley *et al.*, 2004) and co-clustering of biological networks and yeast gene-expression data by combining different distances (Hanisch *et al.*, 2002). Here, we considered two factors for defining functional conservation, namely the similarity in protein sequence and the similarity in the regulation of gene expression, the latter being reflected by co-expression.

The influence of these two factors was accounted for by combining two dissimilarity measures, a measure for protein sequence similarity (δseq) and a measure for gene co-expression (δexp), into a single dissimilarity measure, referred to here as the dissimilarity measure for conservation Δ (Equation 1).

\[
\Delta = \min(\delta_{\text{seq}}, \delta_{\text{exp}})
\]

that describes functional conservation as modules containing four genes (Fig. 1A). To apply this formula to the comparison of one organism (geneset X) to another (geneset Y), we considered gene pairs showing a similar protein sequence (x,y) and (x’,y’) and gene pairs showing co-expression (x,x’) and (y,y’). The function δexp : X × Y → [0, 1] is defined below:

\[
\delta_{\text{exp}}(x,y) = \begin{cases} t \cdot |\text{score}(x,y)|^{-1} & \text{if } score \geq t \\ 1 & \text{otherwise,} \end{cases}
\]

where score(x,y) is an arbitrary, positive, sequence pair alignment scoring function, and t is the threshold. Distances between expression patterns are usually calculated with a Pearson correlation coefficient ρ. As we were only interested in similar expression patterns, we introduced a positive threshold ts. The function δseq : X × X → [0, 1] is defined below:

\[
\delta_{\text{seq}}(x,x') = \begin{cases} 1 - \rho(x,x') & \text{if } \rho \geq ts \\ 1 & \text{otherwise.} \end{cases}
\]

We then computed Δ using a function that (1) accounts for the influence of protein sequence similarity and that of gene co-expression with no a priori, and (2) results in a dissimilarity measure that may be used as an input for data clustering. One function that fulfills these requirements and is capable of balancing δseq and δexp equally, is the sum of the two dissimilarity measures. The function Δ : (X × Y) × (X × Y) → [0, 4] is defined below:

\[
\Delta((x,y),(x',y')) = \begin{cases} 0 & \text{if } x = x' \text{ and } y = y' \\ \delta_{\text{seq}}(x,y) + \delta_{\text{seq}}(x',y') + \delta_{\text{exp}}(x,x') + \delta_{\text{exp}}(y,y') & \text{otherwise.} \end{cases}
\]

As Δ combines two dissimilarity measures, it is symmetrical and reflexive (the demonstration, not given here, is straightforward), two properties that make it sufficient for use as an input for data clustering.

The focus of the dissimilarity measure combination defined by the function Δ is to compare functional features (here sequences and gene expression) as variables modeled by the evolutionary pressure. This analysis was applied to the comparison of *C.elegans* and *Drosophila* genes as illustrated in Figure 2. First, we defined orthologous relationships between proteins of the two organisms (*The C.elegans Sequencing Consortium*, 1998; *Adams et al.*, 2000). Protein sequences for *C.elegans* were retrieved from the Sanger Institute website (wormpep89), and those for *Drosophila* from Flybase release 3 (Flybase Consortium, 2003). The all-by-all protein comparison was performed using the zval algorithm (Comet *et al.*, 1999; Aude and Louis, 2002) of the Biofacet™ software (Glemet and Codani, 1997) set to default parameters (gapo 50, gape 3, cutoff 220, matrix Dayhoff 10/3). This algorithm computes the Z-value, which is based on a Monte-Carlo approach to estimate the significance of a Smith–Waterman alignment score (Smith and Waterman, 1981). In contrast to alignment scores, Z-values reduce the biases due to sequence length and composition (Comet *et al.*, 1999) and are independent of the size of the database being queried. The zval algorithm has been used for massive analysis of protein families as reported, e.g. in the ChSTI database, an automatic classification of UniProt Knowledgebase proteins into groups of related proteins (Kriventseva *et al.*, 2001). Setting the Z-value threshold to 10 corresponds to a statistical alpha-risk (risk of wrongly concluding that there is a difference when really there is none) of 1% (Bastien *et al.*, 2004). If using the Z-value as a score function, Equation (2) becomes

\[
\delta_{\text{zval}}(x,y) = \begin{cases} 10 \cdot \text{Zval}(x,y)^{-1} & \text{if } \text{Zval} \geq 10 \\ 1 & \text{otherwise.} \end{cases}
\]

and allows a group of 177 944 homologous relationships involving 10 625 (52%) *C.elegans* and 9261 (67%) *Drosophila* proteins to be obtained.

Second, we generated gene expression clusters using one expression data set for *C.elegans* (*Kim et al.*, 2001), and one for *Drosophila* (*Arbeitman et al.*, 2002). The *C.elegans* dataset was generated using cDNA microarrays that contained 17 817 genes and corresponded to 553 experiments involving whole animal RNA (*Kim et al.*, 2001). The *Drosophila* dataset was generated using cDNA microarrays that contained 4028 genes and corresponded to wild-type flies examined during 66 sequential time periods from fertilization to the first 30 days of adulthood using whole animal RNA (*Arbeitman et al.*, 2002). We identified 48 088 homologous relationships involving encoded products for which gene-expression data were available, and corresponding to 6222/10 625 (58.5%) *C.elegans* and 2781/9261 (30%) *Drosophila* genes. We applied hierarchical clustering (Johnson, 1967) to these microarray datasets using the amap package from the R statistical language and environment version 1.8.1 (http://www.r-project.org). We calculated a distance matrix that contained all possible pairs of genes by applying the Pearson correlation coefficient formula. We then computed a hierarchical clustering on this matrix, using the Ward’s minimum variance agglomeration method (Ward, 1963). Final gene expression clusters were constructed in two steps. First, we elected to calculate the optimal cluster partition. This was performed using two different validation indices, namely the Dunn’s and Silhouette indices (Bobshakova and Azaoui, 2003). This resulted in 97 clusters containing 155–372 *C.elegans* genes (mean size: 158 genes) and 82 clusters containing 8–185 *Drosophila* genes (mean size: 49 genes). Second, we considered the Pearson correlations (ρ) between genes belonging to the same cluster, and we assessed their statistical significance using Student’s t-test (|t = ρ((n − 2)/(1 − ρ^2)/2)|) with (n − 2) degrees of freedom, where n is the number of genes for each organism. Among the 1 437 118 associations for *C.elegans* and 131 434 associations for *Drosophila*, 21 050 correlations that would occur by chance for *C.elegans* (1.5%) and *Drosophila* (0.6%) had to be excluded for a t-test significance level of 10^−5. The remaining Pearson correlations reflected positively correlated genes, and their value was in the range [0,1], thus allowing δexp values to be in the same range.
Formal representation of dissimilarity measure combination in the BBCP procedure. Functional conservation is inferred, with no a priori, from the influence of protein similarity (dissimilarity measure $\delta_{seq}$) and co-expression (dissimilarity measure $\delta_{exp}$) for genes $x$, $y$, $x'$ and $y'$ (A). When comparing *C. elegans* ($W$) and *Drosophila* ($F$), protein sequence similarity is defined by the Z-value for ($w_A$, $f_A$) and ($w_B$, $f_B$), while co-expressed genes ($w_A$, $w_B$) and ($f_A$, $f_B$) share a Pearson correlation ($\rho$) and are defined by the dissimilarity measure $1 - \rho$ (B).

Flowchart of the BBCP comparative method. Homologous relationships were computed through all-by-all comparison of *C. elegans* and *Drosophila* proteins using the zval algorithm of the Biofacet program (left upper panel). Gene expression clusters were computed from RNA studies using hierarchical clustering, and validated using the Dunn’s and silhouette indices, and Student t-test (right upper panel). Functionally conserved modules (FCMs) were computed with no a priori through calculation of the dissimilarity for conservation $\Delta$ (lower panel).
Finally, the dissimilarity for conservation $\Delta$ was defined between two gene pairs from *C. elegans* (W) and *Drosophila* (F) as

$$\Delta = \delta(w_A, f_A) + \delta(w_B, f_B) + \delta(w_A, w_B) + \delta(f_A, f_B), \quad (6)$$

where $\delta(w_A, f_A)$ applied to proteins encoded by genes $w_A$ and $f_A$, $\delta(w_A, w_B)$ applied to proteins encoded by genes $w_B$ and $f_A$, $\delta(w_A, f_B)$ applied to co-expressed genes $w_A$ and $w_B$, and $\delta(f_A, f_B)$ applied to co-expressed genes $f_A$ and $f_B$ (Fig. 1B). The matrix describing $\Delta$ values was used to compute a hierarchical classification (Johnson, 1967) based on Ward’s agglomeration method. This method iteratively merges the two closest clusters, resulting in a binary tree. As the matrix describing $\Delta$ values was sparse, we used a modified ascending hierarchical classification method and obtained a set of trees, referred here to as functionally conserved modules (FCMs). Overall, the underlying idea of the BBCP method was thus to extract more functionally relevant data on genetic conservation by considering sequence similarity and gene co-expression at the same level.

Validation analysis of functionally conserved modules

To evaluate the biological significance of FCMs, we used GO annotations (Ashburner et al., 2000). Gene ontology data were downloaded from the GO consortium website (http://www.geneontology.org) in March 2004. We sought to identify statistically significant link(s) between GO terms and FCM gene content. The probability of selecting the observed number of genes from a given GO category by chance was calculated using the hypergeometric distribution. This calculation takes into account the total number of genes in all FCMs ($N$), the number of genes in the FCM considered ($n$), the number of genes in a particular GO category ($M$) and, in the FCM considered, the number of genes defined by the GO category ($m$):

$$P\text{-value} = \frac{C^M_n + C^{N-M}_n}{C^N_m}. \quad (7)$$

This probability is strongly dependent on the abundance of annotations available in each of the FCMs. Thus, associations that were not statistically significant may reflect poor annotation for the FCM genes. In addition, we sought to identify statistically significant link(s) between the FCM gene content and RNAi phenotypes from large-scale RNAi screens (Ashrafi et al., 2003; Kamath et al., 2003; Simmer et al., 2003; Boutros et al., 2004; Nollen et al., 2004) by using the hypergeometric probability.

As a complement to using GO annotations and RNAi phenotypes, we used InterPro protein domain annotations (Mulder et al., 2003) downloaded from the European Bioinformatics Institute website (http://www.ebi.ac.uk/interpro) in June 2004. We sought to identify FCMs that primarily described a single protein family or that comprised two or more different protein domains as carried by different proteins. Additionally, we used functional descriptors for microarray studies of peculiar physiological responses or signaling pathways (De Gregorio et al., 2002; Gaudet and Mango, 2002; Klebes et al., 2002; Mallo et al., 2002; Romagnolo et al., 2002; Zhang et al., 2002; Lee et al., 2003; Wang and Kim, 2003; Roesthroem-Lindquist et al., 2004). Finally, we analyzed the scientific literature for the FCMs found.

**RESULTS**

Balancing protein similarity and gene co-expression reveals new conserved genetic modules

We obtained 719 FCMs (Supplementary Figure 1) that involved 1925 gene pairs. Compared to previous studies (Stuart et al., 2003; Bergmann et al., 2004), these conserved modules mostly contained less than eight genes (Fig. 3), and their content was analyzed for GO terms. Although some GO terms may not be very informative, GO is recognized as a useful reference system for the biological classification of large datasets (Stuart et al., 2003; McCarroll et al., 2004).

The examination of FCM content for GO terms indicated that 1578 (82%) *C. elegans* and 1662 (83%) *Drosophila* genes were defined by one or more GO annotations. Statistically significant enrichment in GO terms was observed for 518 (72%) FCMs, as indicated by the association to one or more GO annotations at a significance level of $10^{-7}$ (Supplementary Table 1). This included 65% of the FCMs showing a ‘biological process’ annotation, 84% showing a ‘molecular function’ annotation and 28% showing a ‘cellular component’ annotation (Fig. 4), which indicated a significant coverage of FCMs by GO terms. In each of these ontologies, general-to-precise GO terms were found, suggesting instructive coverage of the FCMs by these GO terms. We observed that FCMs may differ from the modules previously reported to be conserved in *C. elegans* and *Drosophila* (Stuart et al., 2003; Bergmann et al., 2004) by one or more gene(s), or by one or more pair(s) of homologous and/or co-expressed genes. This observation led to the assumption that FCMs containing at least 25% of previously undescribed gene associations may be considered as providing significantly new information. This feature was present in 94% of the conserved modules, 150 FCMs being completely new (Supplementary Figure 1).

Conserved genetic modules for cell type-specific signaling

Using GO terms, we scored 46 FCMs that were almost or fully described in previous studies (Supplementary Table 2) and that corresponded primarily to housekeeping biological processes. For example, we detected modules associated with the proteasome (FCM 102), respiratory chain complex (FCM 193), ribosome (FCM 320), RNA polymerase II complex (FCM 330) and cell cycle (FCM 650). This provided evidence that our approach was able to replicate previous findings on modules that relate to biological processes known to be highly conserved (Teichmann and Babu, 2002; Stuart et al., 2003; van Noort et al., 2003; Bergmann et al., 2004).

Next, we analyzed GO terms for the 50 FCMs that best-ranked for ‘biological process’ and ‘molecular function’ (Supplementary Table 3). While these FCMs appeared to be primarily enriched in housekeeping biological mechanisms, we noticed they may correspond to differentiated functions such as e.g. ‘neuropeptide
Fig. 4. Distribution of GO terms in FCMs. Shown here are the numbers of FCMs significantly described \( (P < 0.01) \) by the GO categories ‘biological process’ (A), ‘molecular function’ (B) and ‘cellular component’ (C). In this figure, the GO terms shown are the most general terms, corresponding to levels 1–3 of the GO hierarchy (Ashburner et al., 2000). The most precise GO term(s) relating to each of the FCMs are indicated in Supplementary Table 1.

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receptor activity’ (FCM 210), ‘synaptic transmission’ (FCM 419) or ‘gametogenesis’ (FCM 622). A more precise analysis of FCM enrichment in GO terms led to the identification of 57 FCMs enriched in differentiated functions (Supplementary Table 4). Our analysis of the scientific literature led to the identification of 75 additional FCMs of this type (Supplementary Table 4). Thus, in addition to detecting FCMs for housekeeping function, the BBCP procedure highlighted a significant proportion (132/719 FCMs: 18%) of conserved modules that described cell type-specific processes, most of these modules (125 FCMs) containing significantly new information. Compared to GO terms describing these modules, we identified a more precise functional and/or developmental annotation for 90/132 FCMs (Supplementary Table 4). Finally, 283 predicted genes were found to be associated with cell type-specific events, illustrating the predictive value of the BBCP method.
The best-balance constraint procedure

**Fig. 5.** Examples of FCMs with high biological content. High biological content is assessed based on analysis of the functional attributes and scientific literature. The FCM 157 contained genes involved in Ras neuronal pathway. FCM 211 contained genes involved in gametogenesis. FCM 238 contained transcription factors or regulators active in germ or neuronal cells. FCM 503 was composed of muscle cell components. FCM 622 was enriched for genes involved in RNA processing and embryogenesis or gametogenesis. m, microarray data; r, RNAi phenotype.

**Conserved genetic modules for precise cell fate specification and physiological activities**

The comparison with genome-wide RNAi knockouts in *C. elegans* (Ashrafi et al., 2003; Kamath et al., 2003; Simmer et al., 2003; Nollen et al., 2004) and *Drosophila* (Boutros et al., 2004) indicated that 350 FCMs contained at least one gene for which a RNAi phenotype was available. Statistically significant enrichment in RNAi phenotypes (Supplementary Table 5) was observed in 7.2% of the FCMs (52 modules). This small percentage was expected as RNAi phenotypes are not available for a large proportion of *C. elegans* or *Drosophila* genes. The observation of RNAi-enriched FCMs supported the notion defined above that the BBCP method identifies functionally conserved gene pair modules. Modules describing cell type-specific signaling (Supplementary Table 4) were indeed observed among the 52 FCMs, e.g. FCM 354, a module that relates to vulva development in *C. elegans* and formation of imaginal disks in *Drosophila*. The occurrence of vulva-related RNAi phenotypes for all worm genes in this FCM notably indicated that the worm D2021.1 predicted gene may participate in the regulation of transcription together with the Zn-finger domain protein flectin flt-1 and deacetylase dcp-66. Furthermore, 29 FCMs (Supplementary Table 6) contained at least one RNAi phenotype associated with lethality in *C. elegans* (‘embryonic lethal’ phenotype) and *Drosophila* (‘lethality’ phenotype), suggesting common survival functions in this FCM group and several new players (predicted genes) to participate in this conserved function.

Using InterPro annotations (Mulder et al., 2003), we detected 29 FCMs strongly enriched for a single protein domain, suggesting the consistent detection of a single family or superfamily of genes by the BBCP method (Supplementary Table 7). For instance, (1) FCM 645 contained three *Drosophila* cytochrome P450 genes classically involved in detoxification, suggesting that the three *C. elegans* predicted proteins may achieve a similar function, (2) FCM 712 corresponded to laminins involved in morphogenesis and (3) FCM 717 contained two *C. elegans* helicases active in the germ line that were associated with two *Drosophila* predicted proteins known to contain helicase domains (Supplementary Figure 1). The use of InterPro annotations also indicated how protein domains were distributed in the FCMs, providing a criterion to detect FCMs that may consistently bring together different ‘molecular function’ categories in terms of signal integration during development. For instance, at the intersect of FCMs showing a ‘spectrin’ domain (8 FCMs) and those showing a ‘myosin’ domain (7 FCMs) were FCM 503 (see the next subsection, and Fig. 5) and FCM 32, a module that contains two pairs of homologous genes associated with epithelial cell morphogenesis. Another example related to protein domains found in transcription factors. We observed a ‘ligand-binding domain of nuclear hormone receptor’ in nine FCMs, a Zn-finger domain in 85 FCMs, and a ‘homeobox’ domain in 24 FCMs. The latter group comprised FCM 631 that brought together the *C. elegans* pair of homeobox genes and the *C. elegans* homeobox genes *ceh-8* (FCM 359), *ceh-17* (FCM 471), *ceh-22* (FCM 423), *ceh-24* and *ceh-26* (FCM 616), *ceh-27* (FCM 467), *ceh-33* (FCM 421) and *ceh-39* (FCM 258). Notable of these was FCM 471 that associated the *C. elegans* paired-like homeobox gene *ceh-17*, a likely ortholog of mammalian *Phox2a/b* expressed in five head neurons (Pujol et al., 2000), with the neuronal...
marker ser-2, the *C.elegans* tyramine receptor gene (Tsailik et al., 2003). The *Drosophila* genes associated in this FCM were the *eyeless* homeobox gene and *Oamb*, the octopamine receptor gene (Han et al., 1998). Tyramine is the chemical precursor of octopamine which is believed to be the invertebrate counterpart of norepinephrine, all being neuroactive substances (Tsailik et al., 2003). This FCM together with overlap of ceh-17 and ser-2 expression in SIA V worm neurons (Pujol et al., 2000; Tsailik et al., 2003) suggested a previously undetected link between ceh-17 and the specification of neurons that express biogenic amine receptors. Additionally, this FCM suggested an overlap of *eyeless* and *Oamb* developmental function, consistent with the notion that these two genes regulate the development of the *Drosophila* mushroom body (Han et al., 1998; Noveen et al., 2000).

To evaluate further the presence of an instructive biological content in the FCMs, we sought to examine the correlation between FCM genes and microarray studies of specific physiological (De Gregorio et al., 2002; Mallo et al., 2002; Zhang et al., 2002; Lee et al., 2003; Roxstrom-Lindquist et al., 2004) or developmental (Gaudet and Mango, 2002; Klebes et al., 2002; Romagnolo et al., 2002; Wang and Kim, 2003) processes in *C.elegans* or *Drosophila*. This evaluation highlighted 363 FCMs containing at least one gene characterized in one of these studies. In this FCM subgroup were highlighted conserved gene sets that may underlie different physiological responses in *C.elegans* and *Drosophila*. For example, we detected 15 FCMs containing both dauer gene(s) in *C.elegans* (Wang and Kim, 2003) and immune response gene(s) in *Drosophila* (Roxstrom-Lindquist et al., 2004). Also highlighted were gene sets that may underlie similar physiological responses in *C.elegans* and *Drosophila*. For example, we detected four FCMs (FCMs 141, 356, 408 and 564) containing immune response genes in *C.elegans* (Mallo et al., 2002) and *Drosophila* (De Gregorio et al., 2002; Roxstrom-Lindquist et al., 2004).

Altogether these observations identified BBCP products as informative and functionally conserved gene modules. Furthermore, our data indicated that precise functional categories such as those involved in cell fate mechanisms and physiological responses may be detected by the BBCP procedure. These observations were pertinent to FCMs that did not appear to fall into conserved functional categories previously detected by means of computational analysis (Stuart et al., 2003; Bergmann et al., 2004).

**Predictive value of BBCP products**

We observed that previously undescribed biological role(s) were suggested for FCM genes. This applied noticeably to predicted genes in FCMs describing cell type-specific activities (Supplementary Table 4). This also applied to a group of 215 predicted genes in FCMs that contained a significant amount of new gene associations and were strongly associated ($P < 10^{-3}$) with a GO term ‘Biological Process’ (Supplementary Table 8). Detailed examples of the predictive value of BBCP products are given below. FCM 157 may define Ras activation in neural lineage (Supplementary Table 4, Fig. 5). This FCM contained the *C.elegans* gene let-60 and two of its transcriptional targets *unc-119* and *F47B10.7* (Romagnolo et al., 2002), let-60 being known to play a role in the differentiation of several *C.elegans* tissues, among which is the neural lineage. Furthermore, *unc-119* is required for nervous system maintenance in *C.elegans* and *Drosophila* (Knobel et al., 2001), and predicted *F47B10.7* is known to be expressed in touch receptor neurons (Zhang et al., 2002). FCM 211 (Supplementary Table 4, Fig. 5) suggested that the predicted worm gene *F17A9.6* may be involved in gametogenesis. This FCM comprised 7/8 genes known to participate in this process, e.g. wee required for oocyte development and maturation (Lamitina and L’Hernault, 2002), and somatically expressed cut, required for germ cell integrity (Jackson and Blochinger, 1997). FCM 238 (Supplementary Table 4, Fig. 5) suggested that the four *C.elegans* predicted genes *C07H6.4, T07F8.4, F35H8.3* and *C14B1.4* may be transcription factors or regulators active in germ or neuronal cells as all *Drosophila* genes in this cluster [Althambra, MTA1-like, Doa (Darkener of apricot), cg (combgap), Taf80 (TBP-associated factor 80 kDa), *Dp* (DP transcription factor)] were known to be involved in these processes, noticeably *Dp* (Cayirlioglu et al., 2003). FCM 503 (Supplementary Table 4, Fig. 5) suggested that predicted *C.elegans* *F21C10.7* and *Drosophila* CG18019 genes may be associated with muscle organization as they were aggregated with three gene pairs encoding myosin heavy chain, paramyosin and tropomyosin (Honda and Epstein, 1990). Finally, FCM 622 (Supplementary Table 4, Fig. 5) suggested a function for seven *C.elegans* and one *Drosophila* predicted genes, here in RNA processing and embryogenesis of gametogenesis. These molecular function and/or biological processes are indeed known to involve 6/7 *Drosophila* genes part of this FCM, e.g. the *Nop* gene family (Vorbruggen et al., 2000).

**DISCUSSION**

The determination of conserved gene sets that may underlie essential developmental and physiological programs using the computational analysis of gene expression is an emerging aspect in comparative biology and, more widely, evolutionary developmental biology (Rast, 2003). One approach in this field aims at studying the conservation of cis-regulatory control circuits in development by modeling ‘gene regulatory networks’ (GRNs) (Bolouri and Davidson, 2002). This approach has noticeably led to the identification of GRNs architecture across 500 million years of echinoderm evolution (Himman et al., 2003). Another approach relies more specifically on inferring functional relationships between conserved genes by exploiting genome-wide data, noticeably microarray data. Pioneer work using microarray data has identified several genes that may act through conserved mechanisms among distantly related organisms (Teichmann and Babu, 2002; Stuart et al., 2003; van Noort et al., 2003; Bergmann et al., 2004; McCarroll et al., 2004). From these studies, there appears to be a tendency for genes in a functional class to be co-expressed, especially those in ancient, permanent or stable complexes (Teichmann and Babu, 2002; Stuart et al., 2003; van Noort et al., 2003; Bergmann et al., 2004), a trend also delineated by studies of co-orthologs as based on genome sequence analysis (Koonin et al., 2004). By balancing protein similarities and gene co-expression, we obtained modules conserved in *C.elegans* and *Drosophila* that essentially differ from previously described clusters (Stuart et al., 2003; Bergmann et al., 2004) in their size and gene content. These differences are likely to reflect the best ability of the BBCP method to identify genes involved in common functions compared to previous approaches. We indeed generated a single subspace of homologous proteins above a given threshold, which is more permissive compared to the best reciprocal hit procedure, and a series of gene expression clusters, here derived from stringent clustering. Balancing protein similarities and gene co-expression with no a priori when calculating genetic conservation (Fig. 2) generated the shortest ‘functional distance’ between genes. Thus, gene association based
on BBCP differed from considering either one of the parameters alone—distance for sequence similarity or co-expression. As a result, we obtained conserved modules that mostly composed of two to four gene pairs, and observed conservation of gene co-expression for proteins that are associated in stable complexes as well as those that do not form such complexes. A significant proportion of conserved modules were previously undetected.

The source data used in our study may influence BBCP informativity. Since all genes were not represented in the microarrays either for C.elegans (Kim et al., 2001) or for Drosophila (Arbeitman et al., 2002), FCM construction may not be optimal for some gene families. Nonetheless, this was unlikely to be a significant problem as nearly all genes are represented in the C.elegans microarrays (Kim et al., 2001). Another factor that may influence FCM gene content is the relative number of cells that involve a small number of cells (Zhang et al., 2001). Yet another factor that may influence FCM gene content is the relative number of different cell types that are represented in the microarrays. Finally, the use of whole animal RNA may reduce the sensitivity for detection of biological processes that involve a small number of cells (Zhang et al., 2002), and may generate associations between gene pairs that are co-regulated but expressed in different cell types or tissues. However, microarray experiments based on cell type-specific RNA remain scarce. Despite these limitations and potential biases, the comparison of FCM gene content with the biological data available for C.elegans and Drosophila genes indicated that the BBCP method consistently detected new elements of genetic conservation. The fact that two or more homologous gene pairs, not necessarily selected as best orthologs, are co-expressed in C.elegans and Drosophila is a strong indication for the putative participation of these genes in essential developmental programs in these two organisms, whether or not they may ultimately result in a similar physiological function. A large proportion (83.4%) of FCMs contained both predicted and known genes, which, together with moderate FCM sizes, provided an enhanced basis for new hypotheses to be raised on the molecular function and/or biological role(s) of several predicted genes. It is to be noted that, several FCMs fall into ‘precise’ molecular, cellular or physiological categories, previously not detected by means of computational analysis. Some modules related, for example, to cell fate specification and physiological activities in C.elegans and Drosophila, which illustrated the potential power of BBCP at discriminating specific gene aggregation events. These features suggested that the BBCP method is able to describe genetic conservation relative to precise aspects of developmental diversity, an essential question in computational biology. It should be noted that (1) the reliability of BBCP products is not dependent on the size of the resulting modules, small FCMs (two to three gene pairs) being as reliable and informative as larger FCMs, (2) genes in FCMs, and most widely in conserved modules were previously undetected.

Our study is based on proteins from C.elegans and Drosophila whose sequences were available as of October 2002, and relies on one set of microarray data comprising a large number of different experimental conditions for each of the organisms studied (Kim et al., 2001; Arbeitman et al., 2002). More recent data were not used or included due to the time-consuming and labor-intensive nature of the BBCP procedure and its validation. The use of these and newly analyzed biological processes will enable more comprehensive studies into gene sets functionally conserved in C.elegans and Drosophila.

In summary, using a new comparative procedure—BBCP—we identified new conserved genetic modules that may underlie C.elegans and Drosophila development. We detected a significant proportion of modules that highlighted new relationships between genetic conservation and developmental diversity in these two organisms. Our case study shows the potential of BBCP for analyzing large and diverse datasets in order to detect conserved genetic modules that may trigger specific developmental events in distantly related organisms. This approach together with other frameworks for the comparative analysis of genomes (Koonin et al., 2004) and postgenomic data (Ashburner et al., 2000; Davidson et al., 2002; Alter et al., 2003; Shannon et al., 2003; Stuart et al., 2003; Kyoda et al., 2004) may allow evolutionary conservation to be studied on the basis of accurate in silico prediction.

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