Uncovering the co-evolutionary network among prokaryotic genes

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ABSTRACT

Motivation: Correlated events of gains and losses enable inference of co-evolutionary relations. The reconstruction of the co-evolutionary interactions network in prokaryotic species may elucidate functional associations among genes.

Results: We developed a novel probabilistic methodology for the detection of co-evolutionary interactions between pairs of genes. Using this method we inferred the co-evolutionary network among 4593 Clusters of Orthologous Genes (COGs). The number of co-evolutionary interactions substantially differed among COGs. Over 40% were found to co-evolve with at least one partner. We partitioned the network of co-evolutionary relations into clusters and uncovered multiple modular assemblies of genes with clearly defined functions. Finally, we measured the extent to which co-evolutionary relations coincide with other cellular relations such as genomic proximity, gene fusion propensity, co-expression, protein-protein interactions and metabolic connections. Our results show that co-evolutionary relations only partially overlap with these other types of networks. Our results suggest that the inferred co-evolutionary network in prokaryotes is highly informative towards revealing functional relations among genes, often showing signals that cannot be extracted from other network types.

Availability and implementation: Available under GPL license as open source.

Supplementary information: Supplementary data are available at Bioinformatics online.

1 INTRODUCTION

Prokaryotic genomes are highly variable in their size (Koonin and Wolf, 2008; Mira et al., 2002). Comparative genomic analyses revealed that variability in gene content among genomes is a major factor contributing to this size variability (Konstantinidis and Tiedje, 2004; Pal et al., 2005). This substantial variability is mainly the result of gene acquisition via Horizontal Gene Transfer (HGT) (Gogarten and Townsend, 2005) and gene loss, e.g. as a result of reductive evolution (Moran, 2003). Gene content across genomes is compactly represented by phyletic patterns (also known as phylogenetic profiles), in which the presence or absence of each COG (Clusters of Orthologous Genes) in each genome is represented as a 0/1 binary character (see Cohen et al., 2008 for details).

Genes can have correlated evolutionary histories. This may reflect mutual dependency constraints, e.g. when these genes correspond to proteins that are part of a complex. Detecting such co-evolutionary interactions is important for understanding genome evolution as a coordinated process rather than as a collection of single evolutionary descriptions of each gene.

For protein and RNA evolutionary studies, a great deal of effort was invested in developing tools that can detect co-evolving sites (e.g. Ashkenazy and Kliger, 2010; Valencia and Pazos, 2002), the most accurate of these explicitly account for the phylogenetic tree that generated these sequences (Duheil et al., 2005; Pollock et al., 1999; Poon et al., 2007). These approaches, in essence, search for correlated evolutionary events among sites, i.e. at two co-evolving positions, the substitutions occur in a pattern that is different from that expected by chance for two independently evolving sites. A similar approach can be used for the detection of co-evolving genes, in which gains and losses tend to co-occur in the same lineages. Accordingly, perfectly correlated genes are co-gained and co-lost during their entire history.

Methods to search for co-evolving genes were previously developed (Dutkowski and Tiuryn, 2009; Ettorna et al., 2001; Glazko and Mushegian, 2004; Hüyen and Snel, 2000; Marcotte et al., 2000; Pellegrini et al., 1999; Wu et al., 2003; Zheng et al., 2002; Zhou et al., 2006). Phylogeny-based examples include methods that employ the maximum parsimony framework (e.g. Campillos et al., 2006; Cordero et al., 2008) and methods that rely on explicit evolutionary models of co-evolution (e.g. Barker et al., 2007). However, using the maximum parsimony criterion may be misleading, in particular when there is substantial variability in branch lengths (Felsenstein, 1978; Pel and Siddall, 2001; Swoford et al., 2001; Yang, 1996). Furthermore, we have previously shown that maximum parsimony is less accurate for the inference of gain and loss events compared with model-based approaches (Cohen and Popko, 2011). Ideally, methods that rely on models that explicitly take into account co-evolution among genes, rather than methods that only use models to test for deviation from the independence assumption should preferably be used. However, explicit modeling of co-evolutionary interactions is currently too computationally extensive to allow the analysis of large datasets, such as those studied in this work.

Here we present a novel probabilistic methodology to detect co-evolutionary interactions from phyletic patterns. We apply our methodology to analyze hundreds of genomes and thousands of COGs and provide novel insights into the co-evolutionary dynamics of genes across the bacterial domain.

2 METHODS

2.1 Evolutionary model and mapping branch-specific events

The input for our methodology includes the phyletic pattern (presence/absence profile) of COGs and the species tree. The phyletic pattern

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was extracted from eggNOG (Muller et al., 2010), an extended version of the COG database (Tatusov et al., 1997). Notably, across the manuscript, we often use term gene as a synonym for the term COG to improve the readability of the text. The analysis is performed on an extensive dataset of 282 prokaryotes. A comprehensive pre-computed microbial species tree was used as input (Dehal et al., 2010; Price et al., 2010). Phyletic pattern data and the input tree are provided as Supplementary Files S1 and S2.

The gain and loss dynamics are modeled as a stationary continuous-time Markov process that allows variability among protein families for both gain and loss rates (Cohen and Pupko, 2010). The model’s free parameters are unknown and are estimated numerically based on the data using the maximum likelihood criterion. Since branch lengths of the input tree are given in units of substitutions per site, we also estimate a branch-length-scaling parameter, which acts to transform all branch lengths to units of gain and loss events.

Given the evolutionary model, for each COG and for each tree branch, the expected numbers of gain and loss events were inferred using the stochastic mapping methodology (Cohen and Pupko, 2010; Minin and Suchard, 2008; Nielsen, 2002).

### 2.2 Simulations-based computation of co-evolution

Let $n$ denote the number of species. Thus, there are $2n-2$ branches in our rooted phylogenetic tree. For a specific COG, the above mapping of events to each branch of the tree can be represented by a vector of size $4n-4$, where half of the entries are used to represent gain events, and the remaining ones represent loss events. We next compute the correlation between the evolutionary histories of a pair of COGs by computing the Pearson’s correlation between the two $4n-4$ dimensional vectors.

The correlation coefficient depends on the number of gain and loss events along the tree. Two COGs that are present in all analyzed species are perfectly correlated; however, this does not necessarily reflect co-evolution. To this end, we only search for co-evolving COGs that experienced a minimal number of gain and loss events across the phylogeny (see also Dutheil et al., 2005). Specifically, we compute for each COG a value, which we term exchangeability, that is the average of the posterior expectation of gain events and loss events across the tree. The distribution of the Pearson’s correlation coefficients was found to highly depend on the minimal exchangeability for independently simulated genes. Pairs with low exchangeability may show extremely high correlations by chance [Supplementary Fig. 51A]. We thus tested for co-evolution only in pairs of COGs with exchangeability above a certain threshold, for both COGs. Too high a threshold may lead to filtering true co-evolving pairs. Too low a threshold would lead to exceedingly high number of pairs tested, in turn may reduce the ability to detect true co-evolving pairs due to multiple testing. We set the exchangeability threshold to five events, to balance between these two considerations.

Positive correlations between two COGs do not necessarily indicate genuine co-evolution, e.g. because high number of events are expected in longer branches for both COGs and vice versa for shorter branches. To test whether an observed correlation value for a given pair is statistically higher than that expected for two independently evolving COGs, we associate each observed correlation coefficient between a pair of COGs with a $P$-value for which the null distribution of the correlation coefficients was computed using parametric bootstrap as follows: for each pair of COGs, we computed the minimum value of exchangeability between them. The observed correlation coefficient for this pair was then compared with a null distribution of independently evolving pairs with comparable minimal exchangeability values, to generate a $P$-value. As stated above, the distribution of correlation coefficients depends on the minimal exchangeability, and thus, the entire range of exchangeability was partitioned into bins, each representing a different null distribution. The bins used are provided in Supplementary Figure S1.

For the size of data analyzed in this study, millions of possible pairs are statistically tested for co-evolution, which necessitates controlling the False Discovery Rate (FDR, Benjamini and Hochberg, 1995). An FDR value of 0.01 was chosen, which let us to consider pairs with $P$-value lower than 1.38E-05.

### 2.3 Analysis of the co-evolutionary network

The network of co-evolving COGs was analyzed and visualized using Cytoscape (Smoot et al., 2011) and Pajek (Batagelj and Mrvar, 2002). The clustering of the network was conducted Transitivity Clustering algorithm (Wittkop et al., 2011) with the default parameters.

### 2.4 Comparing co-evolution network with other biological networks

The protein–protein interactions (PPI), genomic proximity, co-expression and gene fusion networks were extracted from STRING 9.0 (Sklarzyc et al., 2011). For each type of biological association, the network was reconstructed by considering reliable connections between COGs with score of at least 700 (‘high confidence’). The metabolic network was extracted from KEGG by considering metabolic connections between COGs that take part in the same metabolic pathway (Kanehisa et al., 2012).

### 3 RESULTS

#### 3.1 Reconstruction of the co-evolutionary network

Using a phylectic pattern of 4,593 COGs, which are present in at least one of the 282 prokaryotic genomes analyzed, we computed the network of co-evolving genes, in which two vertices (COGs) are connected only if they are significantly co-evolving (Fig. 1; also see Section 2 for details). For 4,933 COGs there are 10,545,528 possible interactions. We only tested for co-evolution among 3,548 COGs with exchangeability above a predefined threshold (see Section 2), which resulted in 6,292,378 pairs.

#### 3.2 Network properties

The network of co-evolving genes was found to be relatively highly connected with an average degree of 3.793. The degree varies substantially among genes in agreement with previous research (Cordero et al., 2008). Out of all COGs, 42.24% (1,940) were found to co-evolve with others (degree $\geq$0). The average degree (3.793) is substantially higher than the median degree (0) suggesting that the network of co-evolution relations does not fit a random network model. Moreover, a goodness-of-fit evaluation for a Poisson degree
We found 3,568 clusters, out of which 2,653 singleton clusters. We have used the transitivity clustering algorithm (Wittkop et al., 1998) was found to be 0.526. This indicates relatively high transitivity in the co-evolutionary interactions. The largest cluster includes 30 COGs. All COGs belonging to the cluster are clearly related for the transcription regulation of the flagellar operon. This co-evolutionary clustering reveals multiple assemblies of genes that have clear modular functional association (see Supplementary File S3 for the complete list of clusters). These results may reflect shortcoming of our methodology or gaps in the current functional annotations.

### Table 1. Clusters of co-evolving COGs associated with specific functions.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Size</th>
<th>Suggested function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>Flagellum and motility</td>
</tr>
<tr>
<td>5</td>
<td>13</td>
<td>NADH:ubiquinone oxidoreductase</td>
</tr>
<tr>
<td>11</td>
<td>9</td>
<td>Cobalamin (vitamin B12) synthesis pathway</td>
</tr>
<tr>
<td>13</td>
<td>8</td>
<td>Molybdenum biosynthesis</td>
</tr>
<tr>
<td>14</td>
<td>7</td>
<td>UDP-N-acetyl processing</td>
</tr>
<tr>
<td>16</td>
<td>7</td>
<td>Hydrolysis of urea</td>
</tr>
<tr>
<td>17</td>
<td>7</td>
<td>FIF1-type ATP synthase</td>
</tr>
<tr>
<td>18</td>
<td>7</td>
<td>Type IV secretory pathway</td>
</tr>
<tr>
<td>22</td>
<td>7</td>
<td>Na-transporting NADH:ubiquinone oxidoreductase</td>
</tr>
<tr>
<td>31</td>
<td>6</td>
<td>Archaeal/vacuolar-type H+ATPase</td>
</tr>
<tr>
<td>33</td>
<td>6</td>
<td>Multisubunit Na+H+ antiporter</td>
</tr>
<tr>
<td>35</td>
<td>6</td>
<td>Type III secretory pathway</td>
</tr>
<tr>
<td>36</td>
<td>6</td>
<td>Cobalamin (vitamin B12) synthesis pathway</td>
</tr>
<tr>
<td>38</td>
<td>6</td>
<td>Mu-like prophage</td>
</tr>
<tr>
<td>40</td>
<td>6</td>
<td>Pilus plus assembly</td>
</tr>
</tbody>
</table>

For example, in *Escherichia coli*, it encodes the FlgA protein: ‘RNA polymerase sigma factor for flagellar operon’.

We observed that in some cases, functional modules were separated into two clusters [e.g. the Cobalamin (vitamin B12) synthesis pathway in clusters number 11 and 36]. Moreover, some clusters do not present a clear functional modularity (Supplementary File S3). These results may reflect shortcoming of our methodology or gaps in the current functional annotations.

### 3.3 Uncovering modular assemblies of genes

We have used the transitivity clustering algorithm (Wittkop et al., 2011) to cluster the network of co-evolution interactions into groups. We found 3,568 clusters, out of which 2,653 singleton clusters (COGs with no significant co-evolutionary partners), 326 clusters of two members, 101 clusters with three members, 42 clusters with four members, 13 clusters with five members and additional 41 clusters with at least six members up to the biggest cluster that consists of 30 COGs. Selected examples with at least six COGs included in the cluster and clear association with specific functions are depicted in Table 1 (See Supplementary File S3 for details).

This co-evolutionary clustering reveals multiple assemblies of genes that have clear modular functional association (see Supplementary File S3 for the complete list of clusters). These members are both highly interconnected and relatively separated from all other clusters (i.e. few connections with genes outside the cluster), suggesting a shared functionality that has relatively low dependency on all other genes.

The largest cluster includes 30 COGs. All COGs belonging to the cluster are clearly related to flagellum functionality and motility (Fig. 3; see Supplementary File S3 for list of COGs and details). For all but one (COG1191), the COG description explicitly states its involvement in flagellum biology. A further inspection of COG1191: ‘DNA-directed RNA polymerase specialized sigma subunit’ revealed that it is also functionally associated with the flagellum. Several genes included in this COG encode for proteins that are related for the transcription regulation of the flagellar operon.

![Degree distribution of the co-evolutionary network on a log-log scale.](image-url)
We next tested the extent to which the co-evolutionary network TrbB (Supplementary Fig. S3). Finally, we used the amino acids expression (e.g. Chen and Dokholyan, 2006), gene fusion (Enright (see Section 2 for details regarding networks reconstruction). These reported to be informative of functional association between proteins overlaps other system-biology related networks that had been order (e.g. Dandekar et al., 1998; Overbeck et al., 1999), co-expression (e.g. Chen and Dokholyan, 2006), gene fusion (Enright et al., 1999; Marcotte et al., 1999; Yanai et al., 2001) and metabolic networks (e.g. Spirin et al., 2006). For the 4,593 COGs in this study, there are 10,545,528 possible connections. For a specific network type we measured the number of reliably reconstructed connections divided by the total number of possible connections (column ‘Frequency’ in Table 2). For a network X and the co-evolutionary network, we additionally computed the number of connections that are both in X and in the co-evolutionary network out of the total number of connection in the co-evolution network (column ‘Conditional frequency’ in Table 2). The highest conditional frequency, 0.173, was computed for the genomic proximity network, indicating that 17.3% of the co-evolutionary connections are also significant genomic proximity connections. In other words, ∼83% of the co-evolutionary edges are not predicted to be genome proximity edges with high confidence. When the genomic proximity network is reconstructed with lower requirement for proximity (low confidence of 150 instead of high confidence of 700 as defined by the STRING database, see Supplementary Table S2 for details), we still find that only ∼36% of the co-evolutionary connections are between neighboring genes. It is clear that genomic proximity substantially increases the probability of co-acquisition and co-deletion of genes and hence it is expected that many co-evolutionary dependencies rise from proximity. However, these results suggest that a major part of all co-evolutionary dependencies may rise between genes that are not in a tight physical proximity.

For each network type, we further compute an enrichment ratio, which is the ratio of the conditional frequency and the frequency. Explicitly, for network type X, the enrichment value approximates the ratio: Pr(edge ? X | edge ? C) / Pr(edge ? X), where C is the set of all co-evolving edges. For example, for PPI, this value is an estimate for the increase in probability for an edge to be involved in PPI when it is known to co-evolve, over the baseline probability estimate for the increase in probability for an edge to be involved in PPI. As shown in Table 2, there is a very high and significant enrichment (461-fold) of the genomic proximity network. The lowest enrichment was observed with the PPI network, indicating that within the co-evolutionary edges, there is only 24.7 fold enrichment for PPI edges. We define functionally informative edges to be edges that connect COGs that share the same functional annotation according to the eggNOG database annotation (Müller et al., 2010). We repeated the analysis above, this time considering only edges that are both between co-evolving COGs and that are functionally informative. The results presented in Table 2 (part B) indicate that the enrichment ratio increases for all network types when considering only functionally informative edges. This suggests that considering only informative edges increases the agreement between the co-evolutionary signal and other system-biology measures. We
We have reconstructed the co-evolutionary network among prokaryotic genes using a novel probabilistic methodology. We suggest that this approach balances well between two objectives. First, the requirement to analyze large datasets of hundreds of genomes, which is currently infeasible with explicit co-evolutionary models (e.g., Barker et al., 2007). Second, the requirement for accuracy and probabilistic inference of significant co-evolutionary interactions, which is lacking when using the maximum parsimony approach (e.g., Corden et al., 2008).

Our results suggest that ~40% of all COGs co-evolve with at least one other member. Furthermore, the inferred co-evolutionary network is significantly enriched in the fraction of connections shared with other cellular network types. Indeed, in previous studies, high agreement between co-evolutionary signal and cellular interaction data was assumed, and hence the cellular interaction data were used as proxy for co-evolution signal (Corden et al., 2008; Fuller et al., 2010). Here, we took a different approach, in which the agreement between cellular interaction data and co-evolutionary signal was not assumed but rather, measured. Our data show that whereas agreement exists, it is far from perfect and the agreement also varies depending on the type of cellular interaction chosen. Although previous studies reported that interacting proteins tend to co-evolve (Juan et al., 2008), we show that it may be misleading to take PPI as proxy for co-evolution, since a small fraction of the co-evolutionary connections are between genes involved in PPI. We note that more research is needed to determine to what extent the lack of overlap between the different networks points to inaccuracies in methodologies and data or to inherent properties of the evolutionary and cellular networks. For example, it is clear the current PPI network is only partially known, especially in bacteria that are evolutionary divergent from model bacterial species such as Escherichia coli.

Whereas in prokaryotes, protein families typically contain only one copy per species (Ranea et al., 2007), in eukaryotes, gene families often comprise hundreds of members. Thus, co-evolutionary signal may reside in gene families that are expanding and shrinking in a correlated manner. Our method only considers presence and absence of COGs, and would hence fail to detect such co-evolutionary pattern. This suggests that birth and death models for gene family sizes should be integrated in co-evolutionary detection method, as was done within the maximum parsimony framework (Corden et al., 2008).

To summarize, we have developed a methodology to infer pairs of co-evolving genes. Using this methodology allows the reconstruction of a co-evolutionary network of COGs. Clustering of the co-evolutionary network reveals many examples of modular assemblies of genes that take part in a specific biological process. Our results suggest that co-evolutionary modules can be a valuable tool in genome annotation efforts.

### 5 ACKNOWLEDGEMENTS

We thank Gil Segal for insightful comments and suggestions, Daniel Yekutieli for his help with the statistic inference and Yaara Oren for critically reading the manuscript.

**Funding:** T.P. is supported by a grant from the Israel Science Foundation (878/09). O.C. and H.A. are fellows of the Edmond J. Safra center for bioinformatics. D.B. is supported by a fellowship from the Israeli Converging Technologies program.

**Conflict of Interest:** none declared.

**REFERENCES**


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**Table 2.** The frequency of interactions within various cellular networks and the conditional frequency of interaction with respect to co-evolutionary interactions.

<table>
<thead>
<tr>
<th>Network type</th>
<th>Frequency</th>
<th>Conditional frequency</th>
<th>Enrichment ratio $P$-value</th>
<th>PPI</th>
<th>Conditional frequency</th>
<th>Enrichment ratio $P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomic proximity</td>
<td>0.000374</td>
<td>0.173</td>
<td>≈ 0</td>
<td>0.000745</td>
<td>0.453</td>
<td>1210</td>
</tr>
<tr>
<td>Gene fusion</td>
<td>0.0000894</td>
<td>0.0186</td>
<td>≈ 0</td>
<td>0.0000894</td>
<td>0.062</td>
<td>693</td>
</tr>
<tr>
<td>Co-expression</td>
<td>0.00161</td>
<td>0.0606</td>
<td>≈ 0</td>
<td>0.00161</td>
<td>0.211</td>
<td>131</td>
</tr>
<tr>
<td>PPI</td>
<td>0.001</td>
<td>0.0248</td>
<td>≈ 0</td>
<td>0.001</td>
<td>0.0972</td>
<td>96.9</td>
</tr>
<tr>
<td>Metabolic</td>
<td>0.00585</td>
<td>0.159</td>
<td>≈ 0</td>
<td>0.00585</td>
<td>0.495</td>
<td>84.69</td>
</tr>
</tbody>
</table>

'Frequency' is the number of connections of that type divided by all possible pairs (10,545,524). 'Conditional frequency' in Table 2, part A is the number of connections within each network type that also co-evolve divided by the total number of co-evolving edges (8,710). In Table 2, part B, 'Conditional frequency' is this number of connections within each network type that are both co-evolving and functional information divided by all co-evolutionary connections that are also functionally informative (1,904). The reported $P$-value is computed against a null hypothesis that there is no enrichment (i.e. the enrichment ratio equals 1) as determined by Fisher’s exact test.


