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 construction 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 (Dutheil 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; Ettema et al., 2000; Dutheil and Davydov, 2007; Glazko and Mushegian, 2004; Hüyen and Snel, 2000; Marotte 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; Pol 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 Pupko, 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
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.

2.1 Simulations-based computation of co-evolution

Let denote the number of species. Thus, there are 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 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 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. S1A). 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, which in turn may reduce the ability to detect true co-evolving pairs due to multiple testing. We set the exchangeability threshold to co-evolve with others (degree ), but 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. S1A). 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, which in turn may reduce the ability to detect true co-evolving pairs due to multiple testing. We set the exchangeability threshold to co-evolve with others (degree ).

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 -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 -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.

3 RESULTS

3.1 Reconstruction of the co-evolutionary network

Using a phyletic pattern of 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 4,593 COGs there are 10,545,528 possible interactions. We only tested for co-evolution among 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 >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
Table 1 (See Supplementary File S3 for details).

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.

3.3 Uncovering modular assemblies of genes

We have used the transitivity clustering algorithm (Wittkop et al., 2011) to cluster the network of co-evolutionary 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 of three members, 42 clusters with four members, 15 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.

For example, in *Escherichia coli*, it encodes the FliA 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 shortcomings of our methodology or gaps in the current functional annotations.

In some cases a cluster with clear function includes genes families that are described as 'Uncharacterized' or 'Poorly characterize' according to the COG annotation. We analyzed in detail two such cases: cluster number 38, related to 'Mu-like prophage' function and cluster number 18, related to the 'Type IV secretory pathway'. Using these examples, we demonstrate the utility of the co-evolutionary network to predict functional annotations.

The 'Mu-like prophage' cluster is composed of the following seven COGs: COG3778, COG4228, COG4379, COG4381, COG4384 and COG4386 (Fig. 4A). For all except one of its members, the COG description explicitly states its association with 'Mu-like prophage' proteins. The COG description for COG3778 is 'Uncharacterized protein conserved in bacteria'. Inspecting the annotation of genes within this COG reveals several examples which suggest that this member is also genuinely related to Mu-like prophage proteins. For example, in *Haemophilus influenzae* and *Vibrio cholerae*, these genes encode 'Mu-like prophage FliMu protein gp48'.

Out of the seven COGs included in the Type IV secretory pathway cluster we found five COGs with COG descriptions that clearly depict them as components of the Type IV secretory pathway (COG5268, COG4959, COG3838, COG3846, COG3701) and an additional one described as "Conjugal transfer/entry exclusion protein" (COG5314), which fits the function of the entire cluster as all genes encode proteins that are part of the Type IV conjugation system. However, COG5489 is described as "Uncharacterized conserved protein". As visualized in Figure 4B, this COG has co-evolutionary interactions with all other six members of the cluster.
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 et al., 2000; Overbeek et al., 1998; Overbeek et al., 2005).]

3.4 The overlap of the co-evolutionary network with other cellular networks

We next tested the extent to which the co-evolutionary network overlaps other system-biology related networks that had been reported to be informative of functional association between proteins (see Section 2 for details regarding networks reconstruction). These include PPI (e.g. Juan et al., 2008), genomic proximity and gene-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. Spivak 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 0.3 instead of high confidence of 0.7 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: $P(\text{edge } X | \text{ edge } X^C) / P(\text{edge } X)$, 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 of 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-evolving 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 (Muller 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
Although previous studies reported that interacting proteins tend to co-evolve (Juan 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 the agreement between cellular interaction data and co-evolutionary signal is significantly enriched in the fraction of connections shared with other cellular networks 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 (Cordero et al., 2008; Tuller 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 evolutionarily divergent from model bacterial species such as Escherichia coli.

Whereas in prokaryotes, protein families typically contain 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 (Cordero 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.

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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.

| Network type       | Frequency | Conditional frequency | Enrichment ratio | P-value | Frequency | Conditional frequency | Enrichment ratio | P-value |
|--------------------|-----------|-----------------------|------------------|---------|-----------|-----------------------|------------------|---------|
| Genomic proximity  | 0.000374  | 0.173                 | 461              | ≈ 0     | 0.000374  | 0.453                 | 1210             | 0       |
| Gene fusion        | 0.0000894 | 0.0186                | 208              | ≈ 0     | 0.0000894 | 0.062                 | 693              | 0       |
| Co-expression      | 0.00161   | 0.0606                | 37.7             | ≈ 0     | 0.00161   | 0.211                 | 131              | 0       |
| PPI                | 0.001     | 0.0248                | 24.7             | 3.2E-211| 0.001     | 0.0972                | 96.9             | 9.02E-286|
| Metabolic          | 0.00585   | 0.159                 | 27.11            | 0       | 0.00585   | 0.495                 | 84.69            | 0       |

‘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 the 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.

We observe that the highest conditional frequency is found for the genomic proximity network: 45.3% of functionally informative co-evolutionary connections are also inferred to be significant genomic proximity connections. Importantly, this result suggests that over half of the functionally informative co-evolutionary connections could not be directly inferred by mere physical proximity. For the PPI network, the conditional frequency with functionally informative co-evolutionary connections is only 9.72%. This suggests that >90% of the functionally informative co-evolutionary connections are between genes that may not be in direct physical interaction (as determined by their relatively low PPI score). Similar results were obtained when the PPI interactions with lower confidence were considered (Supplementary Table S2 provides all the results given in Table 2 (part A) for low and medium confidence for the five network types).

4 DISCUSSION

We have reconstructed the co-evolutionary network among 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. Cordero 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 (Cordero et al., 2008; Tuller 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.
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