
Phylogeny of metabolic networks: A spectral graph theoretical approach

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Many methods have been developed for finding the commonalities between different organisms in order to study their phylogeny. The structure of metabolic networks also reveals valuable insights into metabolic capacity of species as well as into the habitats where they have evolved. We constructed metabolic networks of 79 fully sequenced organisms and compared their architectures. We used spectral density of normalized Laplacian matrix for comparing the structure of networks. The eigenvalues of this matrix reflect not only the global architecture of a network but also the local topologies that are produced by different graph evolutionary processes like motif duplication or joining. A divergence measure on spectral densities is used to quantify the distances between various metabolic networks, and a split network is constructed to analyse the phylogeny from these distances. In our analysis, we focused on the species that belong to different classes, but appear more related to each other in the phylogeny. We tried to explore whether they have evolved under similar environmental conditions or have similar life histories. With this focus, we have obtained interesting insights into the phylogenetic commonality between different organisms.

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1. Introduction

Analysing the commonalities between biological organisms and classifying them is a fundamental approach in evolutionary studies. Organisms are primarily divided into three domains: Archaea, Bacteria and Eukaryote. A traditional way to find phylogenetic relationships between different species is based on similarity in the sequence of orthologous genes, e.g. SSU rRNA (16S rDNA) gene sequence (Olsen *et al.* 1994). It is not easy to identify orthologs and paralogs in the genomes due to gene duplication, gene loss and horizontal gene transfer. Moreover in ‘molecular approach’, protein sequences are also exploited to study the evolutionary relationships (Woese 1998; Doolittle *et al.* 1999). However, the effect of horizontal gene transfer (Doolittle *et al.* 1999; Wolf

et al. 2002), which has been observed in many organisms, on the phylogenetic relationship among species, considering the system level of different cellular functions, is not very clear. Some studies have shown that more than 10% genes in organisms including bacteria, archaea, and eukaryotes are laterally acquired (Garcia-Vallve *et al.* 2000; Ochman *et al.* 2000; Koonin *et al.* 2001; Hedges *et al.* 2002). This supports a strong influence of horizontal gene transfer in cellular evolution (Martin *et al.* 1999; Dutta *et al.* 2002; Jain *et al.* 2002). However, since an organism may have genes from different sources, i.e., may have more than one ancestor, the evolutionary history of organisms can be better represented by a net rather than a tree (Doolittle *et al.* 1999; Martin *et al.* 1999). With ever-growing molecular data, we have many fully sequenced genomes that are very useful for comparative analysis of organisms at a system

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level and which may give us new insights into the process of evolution of organisms that could not be explored by traditional phylogenetic analyses based on a limited number of genes or proteins (Wolf *et al.* 2002).

The annotations of the metabolic reactions contain information regarding cellular activities and their presence in various species (Kanehisa *et al.* 2006). Distance between species can be estimated based on the content of genes encoding enzymes in their genome, or on the metabolic reactions network, or both (links between these two aspects have been explained in the work by Liu *et al.* (2007)). A method to construct a phylogeny, based on enzyme, reaction, and gene content comparison can be found in the work by Ma *et al.* (2004). A phylogeny could be reconstructed by using graph kernel for comparing metabolic network structures (Oh *et al.* 2006). The set-algebraic operations have been used (Forst *et al.* 2006) and the seed compound content has been compared (Borenstein *et al.* 2008) to trace the phylogeny. By computing the distances between the vectors of several network-descriptors estimated on the network of interacting pathways, the cross-species phylogenetic distance could be predicted (Mazurie *et al.* 2008). The study of the co-evolutionary relationships, by comparing the metabolic pathways to the evolutionary relationship between different species based on the combined similarities of all of their metabolic pathways, can be found in the work by Mano *et al.* (2010).

In this work, we perform extensive phylogenetic comparison of 79 completely sequenced organisms (7 eukaryotes, 13 archaea, 59 bacteria) by comparing the structure of their metabolic networks at system level. It is expected that the three domains: Archaea, Bacteria and Eukaryote would be clearly visible. But we focused on the species that come close to each other in our study in spite of belonging to different classes and attempted to find similarity in their life histories. However, our method was not able to strongly demonstrate ‘horizontal gene transfer’ as a reason for this closeness. To investigate the structural similarities between various metabolic networks, we compared spectral density of the normalized graph Laplacian (Banerjee *et al.* 2007) and analysed the biological significance for the species whose spectral density is similar to each other. The spectrum of the normalized graph Laplacian not only reflects global structure of a network but also the local topologies that emerge from different graph operations such as, duplication of a motif (induced subgraph), attachment of a small structure into the existing network (Vukadinovic *et al.* 2002; Banerjee *et al.* 2009, 2015), etc. The distribution of the spectrum was considered as a signature of a network structure, and the spectral plot can distinguish network structure from different sources (Banerjee *et al.* 2008b). The networks constructed from the identical evolutionary processes have similarities in their spectral plots and thus the spectral distance between two

networks can be used to devise a similarity measure of their structures (Banerjee 2012).

2. Methods

2.1 Normalized graph Laplacian spectrum

Let G be an undirected and unweighted network with the vertex set $V=\{i:i=1,\dots,N\}$. If the vertices i and j are connected by an edge, we call them neighbours and it is denoted by $i\sim j$. The number of neighbours of i is called the degree of i and is denoted by d_i .

The normalized graph Laplacian ($N \times N$) matrix (Banerjee *et al.* 2008a) is defined as: $\Delta=(\Delta)_{ij}, i,j=1,\dots,N$ where

$$(\Delta)_{ij} := \begin{cases} 1 & \text{if } i = j \\ -\frac{1}{d_i} & \text{if } i \sim j \\ 0 & \text{else} \end{cases} \quad (1)$$

Note that, this operator is similar with the operator studied in (Chung 1997) but is different than the operator widely studied as (algebraic) graph Laplacian (Mohar *et al.* 1991). Now we can write the eigenvalue equation as $\Delta u - \lambda u = 0$ where the nonzero solution u is called an eigenfunction for the eigenvalue λ . Some of the eigenvalues among N eigenvalues of Δ may occur with higher (algebraic) multiplicity.

The eigenvalues of Δ are real and non-negative and the smallest eigenvalue is always $\lambda_1=0$ for any constant eigenfunction u . The multiplicity of eigenvalue zero reflects the number of connected components in the network. The lowest non-zero eigenvalue informs us how easily one graph can be cut into two different disjoint components. A graph is bipartite if and only if the highest eigenvalue of Δ , $\lambda_N=2$. Moreover, for a bipartite graph the spectral plot is symmetric about 1. For a complete connected graph, all non-zero eigenvalues are equal to $\frac{N}{N-1}$ (see Chung (1997) for the details).

The eigenvalues also reflect the local structures produced by certain graph operations like doubling of an induced subgraph (motif) or joining of another graph (Banerjee *et al.* 2009). For example, duplication of a single vertex (the simplest motif) produces an eigenvalue 1, which is widely observed with higher multiplicity in many biological networks. If we duplicate an edge (i,j) (motif of size two), it generates the eigenvalues $\lambda_{\pm} = 1 \pm \frac{1}{\sqrt{d_i d_j}}$ and if a chain ($i-j-k$) of length 3 is duplicated, it produces the eigenvalues $\lambda = 1, 1 \pm \sqrt{\frac{1}{d_j} \left(\frac{1}{d_i} + \frac{1}{d_k} \right)}$. With the specific value of the degrees of these vertices, the above two motif duplications produce the eigenvalues 1 ± 0.5 and $1 \pm \sqrt{0.5}$ which are also frequently observed in the spectrum of biological and other networks. Joining a small graph Γ (with an eigenvalue λ and

corresponding eigenfunction that vanishes at a vertex $i \in I$ by identifying the vertex i with any vertex of a graph G produces a new graph with the same eigenvalue λ . For example, joining a triangle (which itself has an eigenvalue 1.5) to a graph contributes the same eigenvalue 1.5 to the new graph produced by the joining process (for more details see Banerjee *et al.* (2009)).

2.2 Spectral density, network distance and clustering of species

Now we convolve the spectrum of a network with a kernel $g(x, \lambda)$ and get the function

$$f(x) = \int g(x, \lambda) \sum_k \delta(\lambda, \lambda_k) d\lambda = \sum_k g(x, \lambda_k). \quad (2)$$

Clearly, $0 < \int f(x) dx < \infty$. In this work, we use the Gaussian kernel

$\frac{1}{\sqrt{2\pi\sigma^2}} \exp\left(-\frac{(x-m_x)^2}{2\sigma^2}\right)$ with $\sigma = .01$. Note that, choosing other types of kernels does not change the result significantly. But the choice of the parameter value is important (Banerjee *et al.* 2007, 2009). For small value of the parameter, the density plot contains many random fluctuations and obscures the global features of the network structure; whereas for large value of the parameter, the details become very blurred. Thus, an optimum value .01 of σ is chosen from a range. We compute the spectral density $f^*(x) = \frac{f(x)}{\int f(y) dy}$.

Now, we use the structural distance $D(G_1, G_2)$ between two different networks G_1 and G_2 as:

$$D(G_1, G_2) = \sqrt{JS(f_1^*, f_2^*)}, \quad (3)$$

where $JS(f_1^*, f_2^*)$ is the Jensen-Shannon divergence measure between the spectral densities (of normalized graph Laplacian) f_1^* and f_2^* of the networks G_1 and G_2 , respectively (Banerjee 2012). Jensen-Shannon divergence measure for two probability distributions p_1 and p_2 is defined as $JS(p_1, p_2) = \frac{1}{2}KL(p_1, p) + \frac{1}{2}KL(p_2, p)$; where

$$p = \frac{1}{2}(p_1 + p_2), \quad (4)$$

where $KL(p_1, p_2)$ is the Kullback-Leibler divergence measure for two probability distributions p_1 and p_2 of a discrete random variable X is defined as

$$KL(p_1, p_2) = \sum_{x \in X} p_1(x) \log \frac{p_1(x)}{p_2(x)}. \quad (5)$$

Although, theoretically, there exist isospectral graphs but they are very rare in real networks. Moreover, the isospectral graphs are qualitatively quite similar in most respects.

Now, we construct a distance table for all organisms studied here by measuring the distance $D(G_1, G_2)$ (defined in (4)) between every pair of corresponding metabolic networks (G_1, G_2). The table is used to produce a splits network (Huson 1998), that not only shows different clusters among species, but also reflects the phylogenetic signal in the distance matrix. We use the software package SplitsTree4 (version 4.13.1) (Huson *et al.* 2006) for the construction of splits (network) tree and neighbour-joining tree. To compare our results, another phylogenetic tree is also produced from SSU rRNA sequences of 79 species from NCBI: (<http://www.ncbi.nlm.nih.gov>) and using the software package FigTree v1.4.1 (<http://tree.bio.ed.ac.uk/software/figtree>).

2.3 Data acquisition and processing

We have downloaded the list of enzymes of the 79 completely sequenced organisms (7 eukaryotes, 13 archaea, 7 α -subdivision of proteobacteria, 3 β -subdivision of proteobacteria, 13 γ -subdivision of proteobacteria, 3 δ -subdivision of proteobacteria, 17 low GC content gram positive bacteria, 3 high GC content gram positive bacteria, 1 fusobacteria, 5 chlamydia, 2 spirochete, 2 cyanobacteria, 1 radioresistant, 2 hyperthermophilic bacteria) from regularly updated *KEGG LIGAND* database (<http://www.kegg.jp/kegg/kegg2.html>) (Kanehisa *et al.* 2006, 2014). The recent bioreaction database created by Michael *et al.* (Stelzer *et al.* 2011), which is an updated version of the same constructed by Ma and Zeng (Ma *et al.* 2003), has been used to create the metabolic reaction database for each 79 species. Michael *et al.* (Stelzer *et al.* 2011) have considered several literatures to decide about reversibility of each metabolic reaction in the dataset. They have excluded currency metabolites to make the data more physiological meaningful.

3. Results and discussion

We constructed metabolic networks separately for 79 species. Metabolites were considered as nodes of the network and we connected two metabolites, a substrate and a product of a reaction, by an edge. All constructed networks were not connected; rather, they were composed with a giant component and many isolated small components. In our work, we only considered the giant component. We believe that this part of the network is composed with the most studied metabolic pathways, thus it may be extensively revised and contains more errorless information. Here, we also considered the underlying undirected graph which itself carries adequate structural information for our work. This method

can be easily extended to study a directed network. Now, we used our method to construct a distance table for metabolic networks of all the organisms studied here. This table was used to produce a splits network which can extract phylogenetic signals that were missed in other tree-representations. The splits network shows that the data contained in that matrix has a substantial amount of phylogenetic signal and some parts of the data are tree-like (see Huson (1998) for details). We also constructed a tree by using neighbour-joining method from our distance matrix to visually capture and compare different clusters easily. It was our goal is not to reproduce the phylogenetic tree, but to cluster the species by quantifying the similarity in their metabolic network structures. Then, we analysed the different clusters in the splits network (figure 1) and neighbour-joining tree (figure 2) produced from the distances between the spectral densities (of normalized graph Laplacian) of metabolic networks of 79 organisms studied here (Both of the trees

(figures 1 and 2) do not demonstrate any significant difference in clustering). To compare the clustering from molecular phylogeny, another tree (figure 5) was produced from SSU rRNA sequences, which were highly conserved, of those 79 species. On the one hand, splits network reflected the evolutionary signature in the data, but on the other hand, some of the clusters (in splits-network and the neighbour-joining tree) were different from the same in the tree constructed by SSU rRNA sequences (Robinson-Foulds distance between these two trees was 75). This suggests that two species that cluster together in spite of belonging to different classes, that is, in different clusters in the phylogenetic tree (figure 5) produced by SSU rRNA sequences of 79 species, may have evolved in similar environmental condition or with similar evolutionary histories that make their metabolic networks topologically more similar than others. Next, we explored the commonalities between these species.

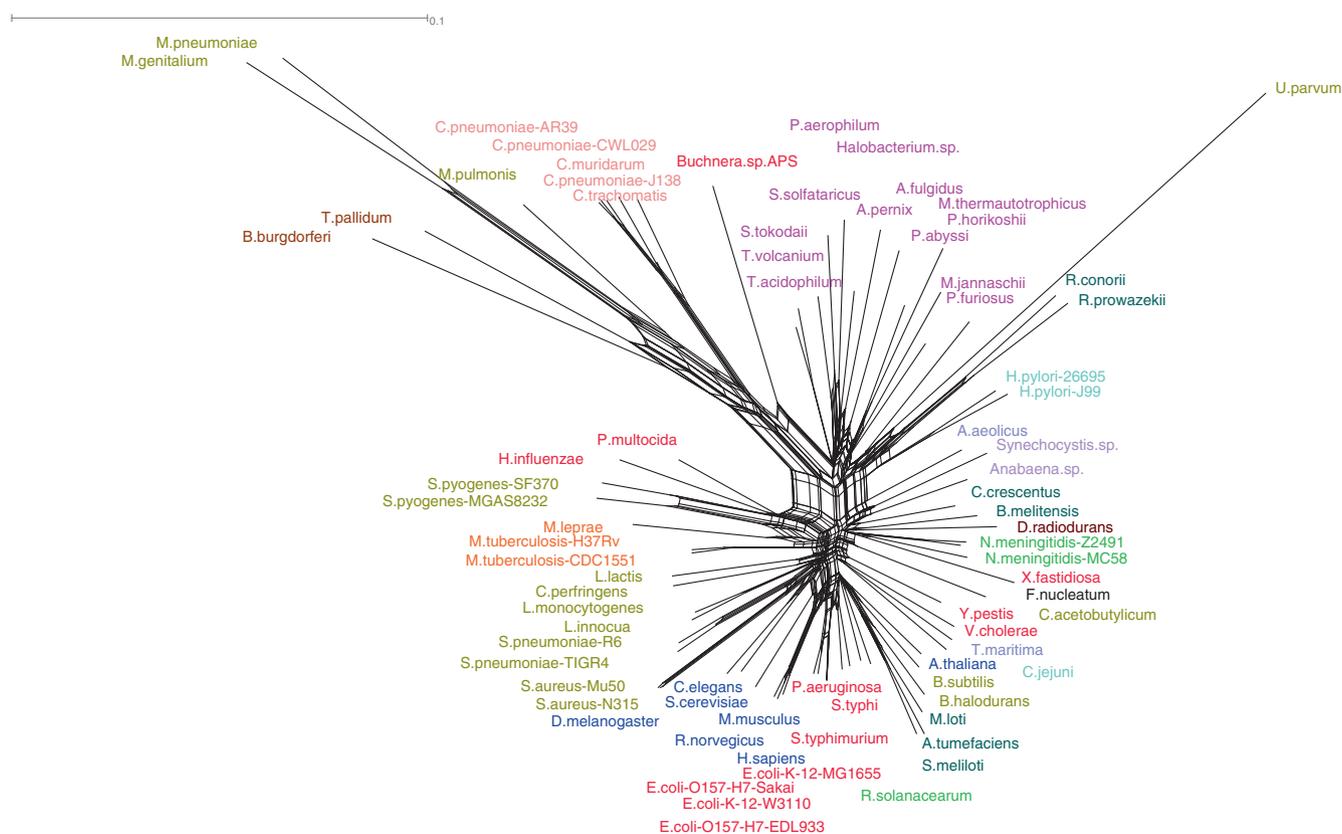


Figure 1. The splits (network) tree constructed from the structural distances between metabolic-centric network of 79 species ■ Eukaryotes; ■ Archaea; and rest of are different subdivision of Bacteria; ■ α -subdivision of proteobacteria, ■ β -subdivision of proteobacteria, ■ γ -subdivision of proteobacteria, ■ δ -subdivision of proteobacteria, ■ Low-GC-content gram-positive bacteria, ■ High-GC-content gram-positive bacteria, ■ Fusobacteria, ■ Chlamydia, ■ Spirochete, ■ Cyanobacteria, ■ Radioresistant bacteria, ■ Hyperthermophilic bacteria.

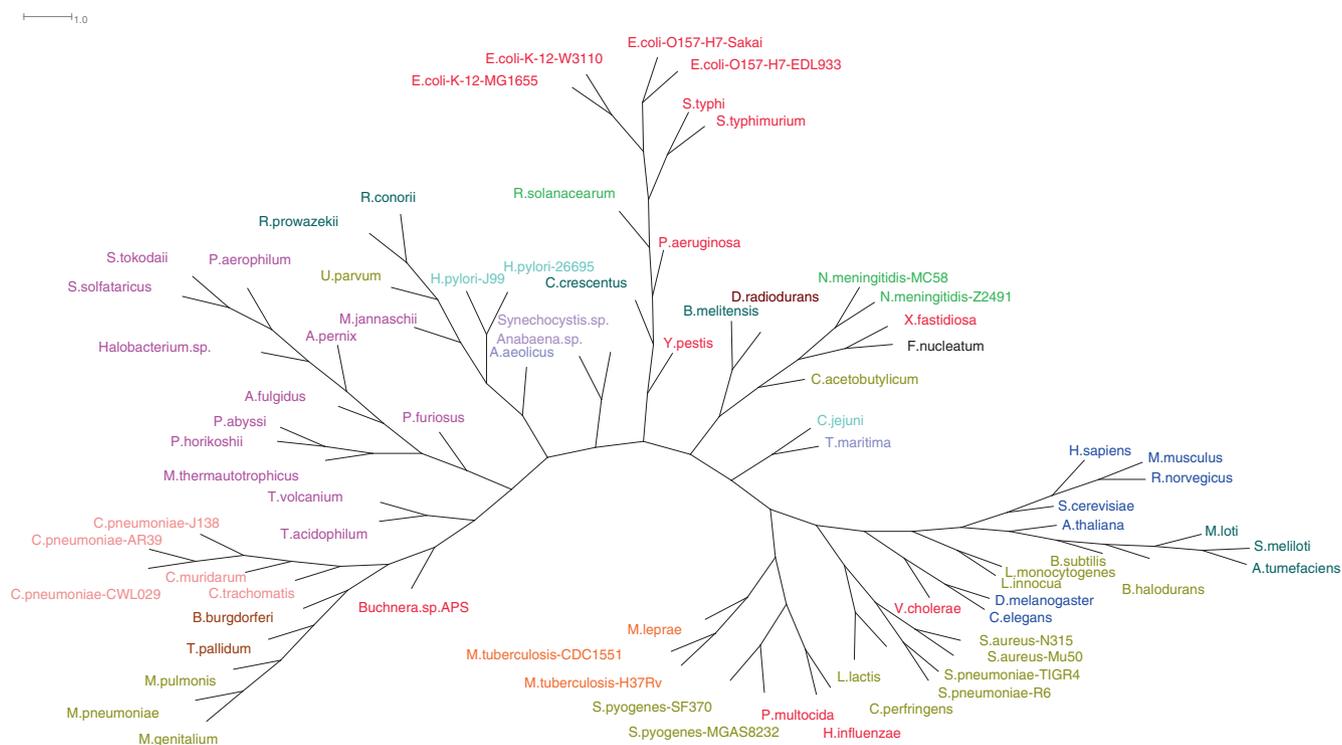


Figure 2. The neighbour-joining tree constructed from the structural distances between metabolic-centric network of 79 species. ■ Eukaryotes; ■ Archaea; and rest of are different subdivision of Bacteria; ■ α -subdivision of proteobacteria, ■ β -subdivision of proteobacteria, ■ γ -subdivision of proteobacteria, ■ δ -subdivision of proteobacteria, ■ Low GC content gram positive bacteria, ■ High GC content gram positive bacteria, ■ Fusobacteria, ■ Chlamydia, ■ Spirochete, ■ Cyanobacteria, ■ Radioresistant bacteria, ■ Hyperthermophilic bacteria.

In our analysis, we observed that *Mesorhizobium loti* and *Sinorhizobium meliloti*, which are two symbiotes, clustered with a free-living (non-symbiote) species *Agrobacterium tumefaciens* of the same group, α -subdivision of proteobacteria. This may be explained by the genome similarity between *S. meliloti* and *A. tumefaciens*, which suggests convergent evolution (see Wood *et al.* (2001) for more details). Moreover, all these organisms are associated with the roots of crop plants (Rudrappa *et al.* 2008).

All the chlamydia species strongly cluster together, which is also observed in the work by Ma *et al.* (2004). One γ -proteobacteria *Buchnera sp. APS* appears near the branch of chlamydia. *Buchnera sp. APS* is endocellular symbiote of aphids. Like parasites, *Buchnera sp. APS* has reduced its genome size and it depends upon host for that of the essential amino acids and vitamins (Shigenobu *et al.* 2000). It reflects that its lifestyle is similar to parasite.

Most of the parasites, from different groups, cluster together. However, the remaining three parasites, *Rickettsia prowazekii*, *Rickettsia conorii* from α -subdivision of proteobacteria and *Ureaplasma parvum*, which is a low-GC-containing gram-positive bacterium, form a separate

branch in our tree. In between these two parasitic branches all archaea cluster together. In the previous studies (Podani *et al.* 2001; Ma *et al.* 2004) which have a slightly different observation, all the parasites cluster together.

Most of the species from γ -subdivision of proteobacteria including *Salmonella typhi*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and four highly related strains of *Escherichia coli* form strongly supported cluster with a lethal plant pathogen from β -subdivision of proteobacteria, *Ralstonia solanacearum*. In whole-genome alignment, *P. aeruginosa* is congruous with *R. solanacearum* (Arodz 2008).

Moreover, like in other methods, all the three high-GC-content gram-positive bacteria clustered together in our tree.

All the above results can also be seen in the tree (splits network and neighbour-joining tree) generated by another method based on a dissimilarity measure using Jaccard-index (Ma *et al.* 2004) on the presence of enzymes in 79 species (figures 3 and 4). Now, we discuss our findings which were not captured by most of the other methods.

Interestingly, our result shows the close relationship between bacteria and eukaryotes that could not captured

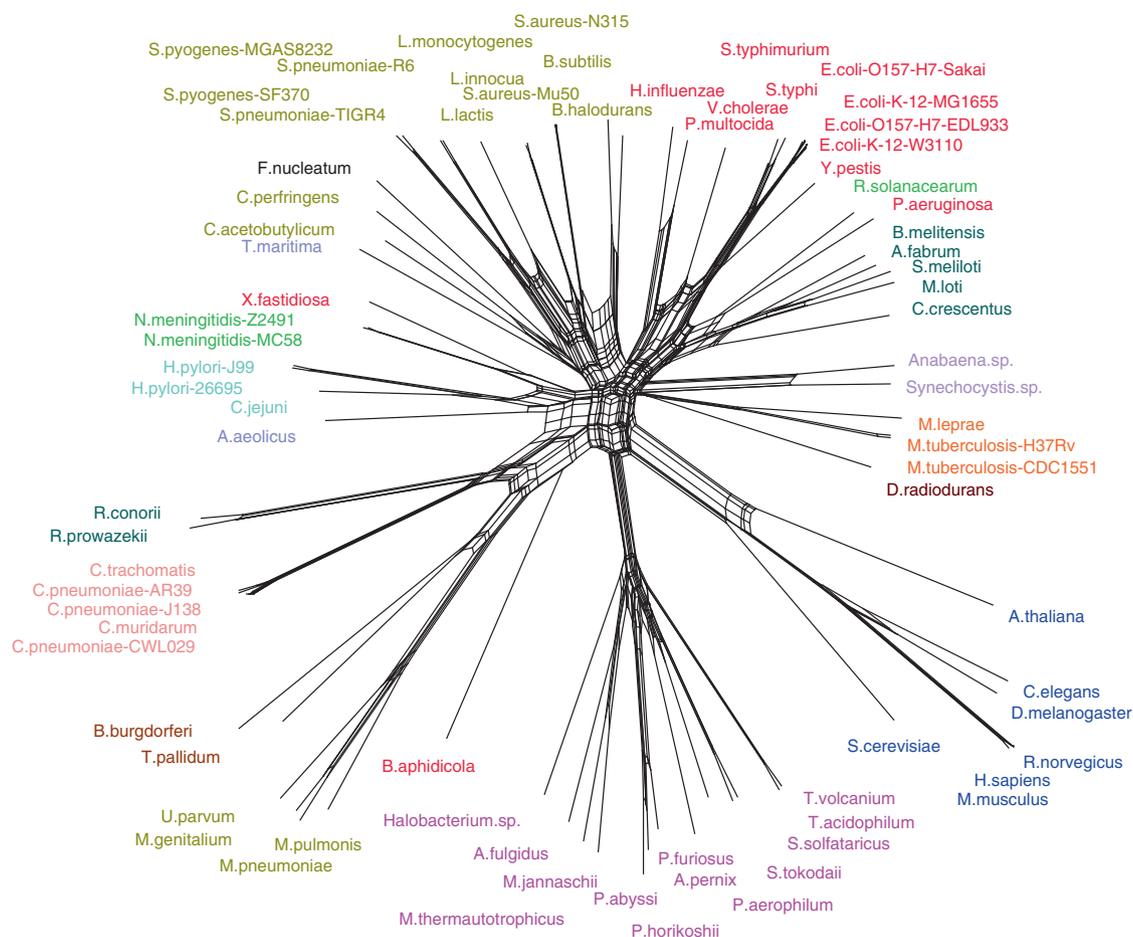


Figure 3. The splits (network) tree constructed from enzymes content of genome-based metabolic networks of 79 organisms with evolution distance based on Jaccard index. Legend: ■ Eukaryotes; ■ Archaea; and rest of are different subdivision of Bacteria; ■ α -subdivision of proteobacteria, ■ β -subdivision of proteobacteria, ■ γ -subdivision of proteobacteria, ■ δ -subdivision of proteobacteria, ■ Low-GC content gram-positive bacteria, ■ High-GC-content gram-positive bacteria, ■ Fusobacteria, ■ Chlamydia, ■ Spirochete, ■ Cyanobacteria, ■ Radioresistant bacteria, ■ Hyperthermophilic bacteria.

by the methods used in the works of Podani *et al.* (2001) and Ma *et al.* (2004). This may happen as most of the metabolic enzymes of eukaryotes are of bacterial origin (Rivera *et al.* 1998).

Six species, from different phylogenetic groups, with similar life histories form a group consisting of a plant in eukaryotes *Arabidopsis thaliana*, two low-GC-content gram-positive bacteria *Bacillus subtilis* and *Bacillus halodurans*, three α -subdivision of proteobacteria *Mesorhizobium loti*, *Sinorhizobium meliloti* and *Agrobacterium tumefaciens*. *B. subtilis*, which is taxonomically related to another alkaliphilic bacterium *B. halodurans*, is a free-living low GC content gram positive bacterium and

associated with the root of the plant *A. thaliana* (Takami *et al.* 2000). As we know, *M. loti* and *S. meliloti* are nitrogen fixation symbiotic bacteria living at the root surface of leguminous plant. But *A. tumefaciens*, which is a free-living pathogenic bacterium, is found in soil and forms biofilm after attaching to the host root (Danhorn *et al.* 2007; Rudrappa *et al.* 2008).

A hyperthermophilic marine bacterium *Aquifex aeolicus* clusters with two cyanobacteria *Synechocystis* and *Anabaena*. All of them participate in nitrogen-fixing (Studholme *et al.* 2000; Tamagnini *et al.* 2002; Makarova *et al.* 2006).

Moreover, our analysis shows that the four species, which are free-living human pathogens that cause respiratory

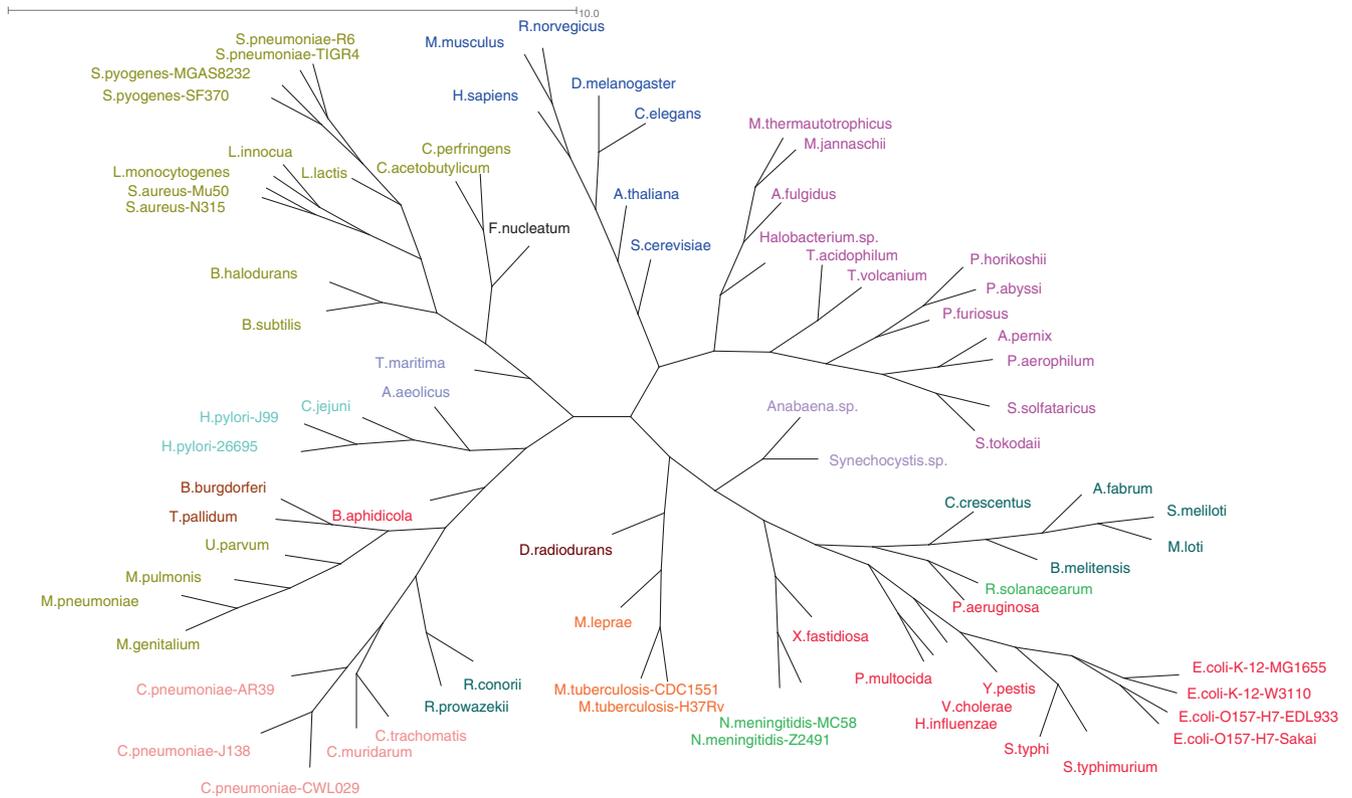


Figure 4. The neighbour-joining tree constructed from enzymes content of genome-based metabolic networks of 79 organisms with evolution distance based on Jaccard index ■ Eukaryotes; ■ Archaea; and rest of are different subdivision of Bacteria; ■ α -subdivision of proteobacteria, ■ β -subdivision of proteobacteria, ■ γ -subdivision of proteobacteria, ■ δ -subdivision of proteobacteria, ■ Low-GC-content gram-positive bacteria, ■ High-GC-content gram-positive bacteria, ■ Fusobacteria, ■ Chlamydia, ■ Spirochete, ■ Cyanobacteria, ■ Radioreistant bacteria, ■ Hyperthermophilic bacteria.

diseases and are from three different groups, appear close to each other in our splits (network) tree. These species are: two γ proteobacteria *Haemophilus influenzae* and *Pasteurella multocida*, one actinobacterium *Mycobacterium tuberculosis*, and a low-GC-content gram-positive bacterium *Streptococcus pneumoniae*. They infect the upper respiratory tract of human. But, there is another low-GC-content gram-positive bacterium *Mycoplasma pneumoniae* which also infects the upper respiratory tract as well as the lower respiratory tract clusters with the parasitic branch.

Evidence shows that the horizontal gene transfer took place between the radioresistant *Deinococcus radiodurans* and γ proteobacteria *Vibrio cholerae* long ago (Eisen *et al.* 2000). They are not very far from each other in our split tree.

Not all the clusters traced by our method are meaningful; for example, one plant pathogen *Xylella fastidiosa* from γ -

subdivision of proteobacteria clusters with *Neisseria meningitidis MC58*, *Neisseria meningitidis Z2491* which are β -subdivision of proteobacteria. The locations of all the clusters of low-GC-content gram-positive bacteria and γ -subdivision of proteobacteria could not be justified. However, many clusters, between various species, found by our study show interesting similarities in some aspects of their lifestyle.

4. Conclusion

Here we have used the spectrum of normalized graph Laplacian that reveals global as well as local architectures, which have emerged from the evolutionary process like motif duplication or joining, random rewiring, random edge



Figure 5. The phylogenetic tree produced by SSU rRNA sequences of 79 species. ■ Eukaryotes; ■ Archaea; and rest of are different subdivision of Bacteria; ■ α -subdivision of proteobacteria, ■ β -subdivision of proteobacteria, ■ γ -subdivision of proteobacteria, ■ δ -subdivision of proteobacteria, ■ Low GC content gram positive bacteria, ■ High GC content gram positive bacteria, ■ Fusobacteria, ■ Chlamydia, ■ Spirochete, ■ Cyanobacteria, ■ Radioresistant bacteria, ■ Hyperthermophilic bacteria.

deletion, etc., of a network. A structural difference measure, based on the divergence between two spectral densities, has been applied to find the topological distances between the metabolic networks of 79 species. A splits (network) tree and a neighbour-joining tree have been used to explore the evolutionary relation between these networks. Our study shows some new interesting insights into the phylogeny of different species, constructed on the basis of their metabolic networks.

The spectrum of non-normalized Laplacian matrix or adjacency matrix can also be used for the same study, but a correlation has been observed between the degree distribution of a network and the spectral density of these matrices (Dorogovtsev 2004; Zhan *et al.* 2010). Due to irregular structure and different sizes, it is difficult to compare different real networks. To investigate the structural similarities, graphs of similar sizes can be aligned to each other. Since for all the networks the spectrum of the normalized graph Laplacian are bound within a specific range (0 to 2), we have an added advantage for comparing spectral plots of networks with different sizes.

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