A global gene evolution analysis on Vibrionaceae family using phylogenetic profile
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Abstract

Background: Vibrionaceae represent a significant portion of the cultivable heterotrophic sea bacteria; they strongly affect nutrient cycling and some species are devastating pathogens.

In this work we propose an improved phylogenetic profile analysis on 14 Vibrionaceae genomes, to study the evolution of this family on the basis of gene content.

The phylogenetic profile is based on the observation that genes involved in the same process (e.g. metabolic pathway or structural complex) tend to be concurrently present or absent within different genomes. This allows the prediction of hypothetical functions on the basis of a shared phylogenetic profiles. Moreover this approach is useful to identify putative laterally transferred elements on the basis of their presence on distantly phylogenetically related bacteria.

Results: Vibrionaceae ORFs were aligned against all the available bacterial proteomes. Phylogenetic profile is defined as an array of distances, based on aminoa cid substitution matrixes, from single genes to all their orthologues. Final phylogenetic profiles, derived from non-redundant list of all ORFs, was defined as the median of all the profiles belonging to the cluster. The resulting phylogenetic profiles matrix contains gene clusters on the rows and organisms on the columns.

Cluster analysis identified groups of "core genes" with a widespread high similarity across all the organisms and several clusters that contain genes homologous only to a limited set of organisms. On each of these clusters, COG class enrichment has been calculated. The analysis reveals that clusters of core genes have the highest number of enriched classes, while the others are enriched just for few of them like DNA replication, recombination and repair.

Conclusion: We found that mobile elements have heterogeneous profiles not only across the entire set of organisms, but also within Vibrionaceae; this confirms their great influence on bacteria evolution even inside the same family. Furthermore, several hypothetical proteins highly correlate with mobile elements profiles suggesting a possible horizontal transfer mechanism for the evolution of these genes. Finally, we suggested the putative role of some ORFs having an unknown function on the basis of their phylogenetic profile similarity to well characterized genes.
Background
Over the past ten years, a great number of microbial genomes have been sequenced covering a wide representation of prokaryotes as well as multiple strains of some species. The study of these genomes both by computational and experimental approaches has highly improved our understanding on physiology, phylogenetic relationship and pathogenicity of many organisms. Furthermore, it has provided new knowledge on microbial genome evolution, revealing a gene core shared by the great majority of bacteria, genes characteristic of particular groups and "novel" genes that possibly originated by lateral gene transfer from some unknown source.

Analysis performed on closely related genomes revealed that a substantial fraction of genes in any genome seem to be strain specific. These genes might sometime arise by gene duplication followed by a rapid divergence, or by lineage-specific loss of genes in one strain, resulting in a unique gene in other strains. However, there are several lines of evidence indicating that lateral gene transfer may be the main mechanism to acquire novel genes. Indeed, this could be one of the main forces driving bacterial adaptation and evolution. Phage DNA is thought to be one of the main vectors for lateral gene transfer among bacteria [1] and many virulence factors from bacterial pathogen are phage encoded [2]. For example, the genes for CT, the most important virulence factor of *V. cholerae*, are encoded in the genome of phage CTXφ integrated in the bacterial chromosome 1.

Since lateral gene transfer plays a relevant role in bacterial evolution, the reconstruction of phylogeny is very complex and phylogenetic trees built by standard sequence analysis may not lead to a reliable picture of the evolutionary history. In fact, alternative trees can be obtained when different proteins are considered.

For many aspects the classification of bacteria on the basis of their global gene content may give a better description of their evolutionary history. This may be particularly important when bacteria of the same group are compared, since newly acquired genes could be relevant to confer peculiar features that allows the exploitation of different ecological niches.

In this study we propose a bioinformatic procedure to investigate bacterial genome evolution, taking into account the global gene content, as well as sequence similarity. We based our analysis on modified phylogenetic profiles [3]; however, we do not consider only the presence/absence of orthologue genes, but also their distance, based on a substitution matrix.

A phylogenetic profile is a non-sequence-homology-based method developed to infer a possible functional relationship between genes. It is based on the idea that proteins that are involved in the same metabolic pathway or structural complex are likely to evolve in a correlate fashion and during evolution appear phylogenetically linked, showing a tendency to be either preserved or eliminated as a whole. Therefore, genes showing similar phylogenetic profiles are likely to be functionally related. We extended the use of phylogenetic profiles to produce an evolutionary tree based on a hierarchical clusterization of organisms with similar phylogenetic profiles.

For this study we took the whole gene dataset of 320 prokaryotic genomes, however, we limited the analysis to the orthologous groups that are present in at least one of the 14 considered species of the *Vibrionaceae* family. These bacteria belong to the *Gammaproteobacteria* group and are highly abundant in aquatic environment, they strongly influence nutrient cycling and various species are also devastating pathogens. Since we focused our analysis on this particular group, the aim of this study is not the construction of a global evolutionary tree, but rather a *Vibrionaceae* perspective of bacterial diversity, based on phylogenetic profiles.

Results and discussion

Phylogenetic matrix

The analysis was performed on 14 bacteria belonging to the *Vibrionaceae* family (Table 1). The redundant list of *Vibrionaceae* ORFs was clustered to reduce the number of proteins to analyze and the phylogenetic profile for each cluster was calculated as described in the Method section.

Many authors proposed and successfully applied different measure methods to calculate the phylogenetic profile values.

Pellegrini *et al.* [3] firstly proposed a phylogenetic profile described as a string of bits, each bit representing the absence or presence of an homologous gene in a given genome. This method lacks a weighting procedure, giving the same weight (value 1) to all the sequences that are considered homologous given a similarity threshold. Enault and colleagues proposed an improved phylogenetic profile based on a normalized Blastp bit score [4]. This method, compared to the approach implemented by Pellegrini, allows weighting each point of the profile proportionally to the length and the quality of the alignment. Jingchun and colleagues optimized the phylogenetic profiles method by integrating phylogenetic relationships among reference organisms and sequence homology information, based on E-value score, to improve prediction accuracy [5].
The measure index \( I \) proposed in this work is similar to the others described above, taking into account both the quality and the length of the alignment using a substitution matrix. Moreover our approach considers also the total length of the sequences, penalizing good alignments occurring between ORFs having different lengths and taking into consideration that ORFs could differentiate mainly for the presence of functional domains.

The final phylogenetic profile for each cluster was defined as the median of all the profiles belonging to the cluster, named “meta-profile”, which describes the profile of conserved ORFs belonging to an entire family.

Hierarchical cluster analysis
A hierarchical cluster analysis was performed on the entire phylogenetic profile matrix and it was calculated a statistical support based on bootstrap method for the nodes of the columns tree (Fig 1). The branch tree colors represent the bootstrap percentage support. This constitutes a phylogenetic tree based on gene content using Vibrionaceae ORFs as a reference. Genomes belonging to the same taxonomic group tend to cluster together and the ORFs as a reference. Genomes belonging to the same taxonomic group tend to cluster together and the ORFs as a reference. Genomes belonging to the same taxonomic group tend to cluster together and the ORFs as a reference. Genomes belonging to the same taxonomic group tend to cluster together and the ORFs as a reference. Genomes belonging to the same taxonomic group tend to cluster together and the ORFs as a reference. Genomes belonging to the same taxonomic group tend to cluster together and the ORFs as a reference. Genomes belonging to the same taxonomic group tend to cluster together and the ORFs as a reference. Genomes belonging to the same taxonomic group tend to cluster together and the ORFs as a reference. Genomes belonging to the same taxonomic group tend to cluster together and the ORFs as a reference. Genomes belonging to the same taxonomic group tend to cluster together and the ORFs as a reference. Genomes belonging to the same taxonomic group tend to cluster together and the ORFs as a reference. Genomes belonging to the same taxonomic group tend to cluster together and the ORFs as a reference. Genomes belonging to the same taxonomic group tend to cluster together and the ORFs as a reference. Genomes belonging to the same taxonomic group tend to cluster together and the ORFs as a reference.

Clustering and genes distribution, as shown in Fig 2, reveals that the number of clusters and genes shared by the organisms decreases as the number of organisms considered increases. The analysis was performed considering for each cluster profile the number of organisms sharing the same numbers of clusters (and genes). The majority of gene cluster groups no more than 21 species on a total of 320. The highest blue spike corresponds to the higher number of genes shared by 105 groups of 14 organisms. Among these groups, as expected, Vibrionaceae are highly represented. Other species represented are Colwellia psychrerythraea 34H and Shewanella oneidensis, that belong to the Alteromonadales family.

The cluster analysis performed on genes is shown in Fig. 3. From now on, to avoid confusing interpretation between clusters derived from the cluster analysis and cluster derived from the ORFs clustering we will use the term "gene" in place of cluster of ORFs.

The different gradient of color, from bright to dark red, represents decreasing similarity values. The cluster analysis allows the detection of three main groups of genes. The first one (Fig 3, panel B) contains the most conserved and established genes shared almost by all the organisms. These core genes can be defined as the set of all genes shared as orthologous by all members of an evolutionary

### Table 1: List of organisms used in the analysis.

| Organism Name          | N. Genes | Life style | Status   |
|------------------------|----------|------------|----------|
| Vibrio_cholerae        | 3835     | Pathogen   | Complete |
| Vibrio_fischeri_ES114  | 3802     | Symbiont   | Complete |
| Vibrio_parahaemolyticus| 4832     | Pathogen   | Complete |
| Vibrio_vulnificus_CMCP6| 5024     | Pathogen   | Complete |
| Vibrio_vulnificus_Y916 | 4488     | Pathogen   | Complete |
| Photobacterium_profundum_S59 | 5494 | Environmental | Complete |
| Vibrio_cholerae_0395   | 3505     | Pathogen   | Draft    |
| Vibrio_cholerae_MO10   | 3418     | Pathogen   | Draft    |
| Vibrio_cholerae_RC385  | 3221     | Environmental | Draft   |
| Vibrio_cholerae_V51    | 3323     | Pathogen   | Draft    |
| Vibrio_cholerae_V52    | 3378     | Pathogen   | Draft    |
| Photobacterium_profundum_3TCK | 5531 | Environmental | Draft   |
| Vibrio_MED222          | 4590     | Environmental | Draft   |
| Vibrio_splendidus_1280I| 5231     | Pathogen   | Draft    |

This highlights the existence of a high variability among lineages, due to genetic and evolutionary processes such as lateral gene transfer, concerted evolution and gene duplication [6]. In terms of gene content, the organisms more related to the Vibrionaceae belong to the gamma and beta proteobacteria. In particular Alteromonadales, Enterobacteriales and Burkholderiales are closely related to Vibrionaceae, and share the higher number of cluster of genes (average percentage of 20%). As expected, Archea are the most distant group sharing just 3.8% of clusters.
The ORFs belonging to these clusters are predicted to code for the ATP binding subunit of ABC transporters (annotated as ABC-type polar amino acid transport system, ABC-type antimicrobial peptide transport system, ABC-type histidine transport system and ABC-type transport system involved in lysophospholipase L1 biosynthesis). This finding is surprising since this is the first report where these ORFs are assigned to the core genes. Anyway two different explanations can be traced. First, it is known that the ABC transporters represent an essential transport system in the prokaryotes and that their ATP binding subunits are apparently overrepresented compared to the other two subunits (ligand binding and permease subunit) in all genomes sequenced thus far [7]. Second, one organism, *Buchnera aphidicola*, presents these genes with a similarity just below the cut-off used for the analysis, but they have been considered since it is well known that in this mutualistic endosymbiont the accelerated evolution and AT bias affect all its genes, including the 16S rRNA [8,9].

The dataset used for the analysis includes genomes in draft quality (*Vibrio cholerae* 0395, *Vibrio cholerae* MO10, *Vibrio cholerae* RC385, *Vibrio cholerae* V51, *Vibrio cholerae* V52, *Photobacterium profundum* 3TCK, *Vibrio MED222*, *Vibrio splendidus* 12B01). Wrong ORFs prediction or missing genes due to incomplete genome sequences can explain the low number of core genes identified. To avoid such problems we repeated the analysis excluding the draft genomes and thus considering 312 genomes. The results, reported in Table 2, show an increased number of the core genes and in particular ribosomal proteins and tRNA synthetase, as reported by Charlesbois and Doolittle [10]. This could be considered as a sort of “minimal genome” containing the group of genes that are necessary to maintain a free-living organism.

The low number of genes shared by all the organisms can be due to many factors. First of all we used the *Vibrionaceae* ORFs as a reference, limiting the number of genes
we were able to identify. It was further demonstrated that
the core gene size decreases as more genome sequences
are analyzed [10].

Genes that are considered to belong to the core set when
close organisms are compared, are classified as flexible
genes when distantly related genomes are analyzed [6].
Finally, genes within core genomes might be transferred
or replaced, introducing new versions of existing genes
into genomes. Such transfers can replace even highly con-
served genes by non-homologous counterparts but the
advantages provided are difficult to explain. It is also to
take into consideration that many symbiotic and parasitic
bacteria undergo a reduction of their genomes, loosing
many genes required by free-living cell.

The second group (Fig 3, panel C) represents genes shared
mainly among Vibrionaceae and other gamma proteobac-
teria (as Altermonadales, Burkholderiales and Enterobactidi-
dales).

Finally, the third group (Fig. 3, panel D) is composed by
genes that are mainly specific to the Vibrionaceae.

**k-mean cluster analysis and cluster enrichment**
We performed a $k$-means cluster analysis, setting the $k$
value to 14. As shown in Fig. 4, the clusters 3, 4, 11, 13
and 14 contain the higher percentage of genes, accounting
for more that 50% of the total genes, while clusters 9 and
10 contain the lower number of ORFs (3% of genes). The
variance in each $k$-means cluster is very low (Fig. 4),
meaning that the clusters contain genes with compact and
similar profiles. As described in Fig. 4, the majority of the
clusters (1, 2, 3, 4, 5, 6, 7, 9, 12, 13) contains genes with
a similar profile, with the average values (red line) near
zero, except for the presence of some spikes correspond-
tent to an increasing similarity with some isolated organ-
isms. As shown in Table 3, clusters 1, 3, 4, 5, and 9
contains genes that have a high similarity in a small subset
of organisms. The majority of these ORFs are annotated as
hypothetical proteins or phage related proteins. Clusters
8, 10 and 14 present genes shared among almost all the
organisms. In particular cluster 10 is composed by the
core genes described before having an high value of simi-
larly widespread among all the organisms; cluster 8 con-
tains genes shared mainly by gamma proteobacteria and
cluster 14 is composed of genes in common between
Vibrionaceae and Enterobacteriaceae.

A functional annotation has been performed on each gene
cluster using COG (Cluster of Orthologous Genes), KEGG
pathway map and GO databases. For each $k$-mean cluster
the enrichment probability with respect to the total
number of clusters has been obtained with the hypergeo-
metric distribution.

Fig. 5 shows COG enrichment results for each cluster. As
expected clusters represented by conserved genes (cluster
8, 10 and 14) have the higher number of enriched COG
codes, while cluster specific of few organisms are charac-
Figure 3
Two-way hierarchical cluster analysis of the entire phylogenetic profile matrix (panel A). Panel B: dendrogram selection zoom of highly conserved genes shared among all the organisms; panel C: genes conserved mostly among gamma proteobacteria; panel D: genes specific of Vibrionaceae family.
terized by a small number of enriched COGs. The majority of clusters presents COG codes enrichment for S (function unknown), R (poorly characterized) and – (absence of COG code) categories. This is due to the large abundance of unknown and hypothetical proteins presents in the Vibrionaceae proteomes.

It is worth noting that cluster 3, mainly represented by Photobacterium profundum SS9 ORFs, is enriched only by C (Energy production and conversion), L (DNA replication, recombination and repair) and M (Cell envelope biogenesis, outer membrane). Probably the L class overrepresentation is determined by the high number of transposons

Table 2: Core genes shared considering different number of genomes. The table shows the progressive number of genes shared with increasing number of genomes.

| N. of Genomes | Genes Annotation |
|---------------|------------------|
| 300           | 3-phosphoglycerate kinase |
| 300           | ABC-type phosphate transport system, ATPase component |
| 300           | Excinuclease ATPase subunit |
| 301           | Membrane GTPase LepA |
| 301           | Ribosomal protein S5 |
| 302           | ABC-type dipeptide/oligopeptide/nickel transport system, ATPase component |
| 302           | Aspartyl-tRNA synthetase |
| 302           | DNA-directed RNA polymerase, betasubunit/140 kD subunit |
| 303           | ABC-type oligopeptide transport system, ATPase component |
| 303           | Ribosomal protein S17 |
| 303           | Ribosomal protein S3 |
| 303           | ATPase components of various ABC-type transport systems |
| 303           | Ribosomal protein S12 |
| 303           | Ribosomal protein S7 |
| 303           | Ribosomal protein S10 |
| 303           | Ribosomal protein L1 |
| 305           | CTP synthase UTP-ammonia lyase |
| 305           | ABC-type uncharacterized transport system, duplicated ATPase component |
| 305           | Hydroxy methylpyrimidine transport ATP-binding protein |
| 306           | Glycine-serine hydroxymethyltransferase |
| 306           | Ribosomal protein S19 |
| 307           | Signal recognition particle GTPase |
| 307           | Thioredoxin reductase |
| 307           | Enolase |
| 307           | ABC-type transport system involved in resistance to organic solvents, ATPase component |
| 307           | Signal recognition particle GTPase |
| 308           | Ribosomal protein S9 |
| 308           | Seryl-tRNA synthetase |
| 308           | Ribosomal protein L2 |
| 308           | ATP-binding protein |
| 309           | Cysteinyl-tRNA synthetase |
| 309           | ABC-type thiamine transport system, ATPase component |
| 310           | Metal-dependent proteases with possible chaperone activity |
| 310           | Predicted ATPase involved in cell division |
| 310           | Ribosomal protein L11 |
| 310           | ABC-type arginine transport system, ATPase component |
| 311           | ABC-type polar amino acid transport system, ATPase component |
| 311           | ABC-type antimicrobial peptidetransport system, ATPase component |
| 311           | ABC-type histidine transport system, ATPase component |
| 311           | Ribosomal protein S11 |
| 311           | Ribosomal protein L14 |
| 311           | GTPases – translation elongation factors |
| 311           | Predicted ABC-type transport system involved in lysophospholipase L1 biosynthesis, ATPase component |
| 312           | Ribosomal protein L5 |
that are present in the SS9 genome [11]. The role played by these transposable elements in the survival of this deep-sea bacterium is still a matter of debate [12].

In addition *V. vulnificus* YJ016 and *V. vulnificus* CMCP6 (cluster 13) seem to share genes belonging to the enriched COG classes K (Transcription), L and T (Signal transduction mechanisms). It was previously reported an enrichment in genes belonging to the transcription class in the genome of *V. vulnificus* respect to the *V. cholerae* genome [13]. This class is clearly related to the T class and seems to indicate that this organism is able to receive and translate in a transcriptional response specific environmental signals. Despite this, the large majority of the genes in clusters 3 and 13 lacks COG annotation.

Cluster 7, as shown in Table 3, accounts organisms with large genome size (see Table 1). This can explain the fact the this cluster contains almost all the COG class enriched and suggests a more complex and flexible life-style of these organisms compared to the other *Vibrionaceae* members.
KEGG annotation is limited to metabolic or structural complex network and so a reduced number of genes have a KEGG entry. This causes the presence of clusters without enriched map (cluster 2–5, 7, 13, see Fig 5). Also in this case, the clusters presenting the higher number of significant KEGG map are those containing the conserved genes. The most enriched KEGG clusters are cluster 14, 10 and 8 accounting for the majority of the metabolic pathways.

Cluster 1 is enriched for map3080 (type IV secretion system). In fact \textit{V. fischeri} genome contains 10 separate pilus gene clusters, including eight type-IV pilus loci. The presence of multiple pilus gene clusters suggests that different pili may be expressed in different environments or during multiple stages of its development as a symbiont [14].

Cluster 11 is enriched for map3090 (type II secretion system). The type II pathway is conserved among gram-negative bacteria, including many pathogens, and secretes a variety of virulence factors and degradative enzymes [15].

Cluster 9 is enriched for map 00860 (Porphyrin and chlorophyll metabolism). These genes are involved in the

| Cluster ID | Organism | Median value | Standard deviation |
|-----------|----------|--------------|--------------------|
| cluster 1 | Vibrio fischeri ES114 | 0.9 | 0.08 |
| cluster 2 | Photobacterium profundum 3TCK | 0.95 | 0.13 |
| cluster 3 | Photobacterium profundum SS9 | 0.9 | 0.09 |
| cluster 4 | Vibrio splendidus 12B01 | 0.9 | 0.1 |
| cluster 5 | Vibrio paraohaemolyticus | 0.9 | 0.1 |
| cluster 6 | V. cholerae | 0.8 | 0.15 |
| &nbsp; &nbsp; | V. vulnificus YJ016 | 0.58 | 0.24 |
| &nbsp; &nbsp; | V. vulnificus CMCP6 | 0.56 | 0.26 |
| &nbsp; &nbsp; | V. cholerae MO10 | 0.88 | 0.12 |
| &nbsp; &nbsp; | V. cholerae O395 | 0.87 | 0.14 |
| &nbsp; &nbsp; | V. cholerae RC385 | 0.78 | 0.25 |
| &nbsp; &nbsp; | V. cholerae V51 | 0.85 | 0.17 |
| &nbsp; &nbsp; | V. cholerae V52 | 0.86 | 0.15 |
| &nbsp; &nbsp; | Vibrio MED222 | 0.5 | 0.2 |
| &nbsp; &nbsp; | Vibrio splendidus 12B01 | 0.51 | 0.28 |
| cluster 7 | Photobacterium profundum SS9 | 0.48 | 0.29 |
| &nbsp; &nbsp; | V. paraohaemolyticus | 0.55 | 0.27 |
| &nbsp; &nbsp; | V. vulnificus CMCP6 | 0.5 | 0.3 |
| &nbsp; &nbsp; | V. vulnificus YJ016 | 0.54 | 0.28 |
| &nbsp; &nbsp; | Vibrio splendidus 12B01 | 0.65 | 0.22 |
| cluster 9 | Vibrio MED222 | 0.97 | 0.12 |
| cluster 11 | Photobacterium profundum SS9 | 0.4 | 0.28 |
| &nbsp; &nbsp; | V. cholerae | 0.51 | 0.15 |
| &nbsp; &nbsp; | Vibrio fischeri ES114 | 0.43 | 0.28 |
| &nbsp; &nbsp; | V. paraohaemolyticus | 0.54 | 0.19 |
| &nbsp; &nbsp; | V. vulnificus CMCP6 | 0.56 | 0.19 |
| &nbsp; &nbsp; | V. vulnificus YJ016 | 0.57 | 0.19 |
| &nbsp; &nbsp; | V. cholerae MO10 | 0.5 | 0.15 |
| &nbsp; &nbsp; | V. cholerae O395 | 0.51 | 0.14 |
| &nbsp; &nbsp; | V. cholerae RC385 | 0.36 | 0.22 |
| &nbsp; &nbsp; | V. cholerae V51 | 0.42 | 0.2 |
| &nbsp; &nbsp; | V. cholerae V52 | 0.48 | 0.17 |
| &nbsp; &nbsp; | Vibrio MED222 | 0.52 | 0.22 |
| &nbsp; &nbsp; | Vibrio splendidus 12B01 | 0.54 | 0.2 |
| cluster 12 | Photobacterium profundum 3TCK | 0.44 | 0.28 |
| &nbsp; &nbsp; | V. cholerae | 0.41 | 0.44 |
| &nbsp; &nbsp; | V. cholerae O395 | 0.5 | 0.45 |
| &nbsp; &nbsp; | V. cholerae MO10 | 0.48 | 0.45 |
| &nbsp; &nbsp; | V. cholerae RC385 | 0.28 | 0.4 |
| &nbsp; &nbsp; | V. cholerae V52 | 0.35 | 0.43 |
| cluster 13 | V. vulnificus CMCP6 | 0.71 | 0.4 |
| &nbsp; &nbsp; | V. vulnificus YJ016 | 0.77 | 0.35 |
cobalamin (coenzyme B12) biosynthetic pathway [16]. Some organisms, such as Salmonella typhimurium and Klebsiella pneumoniae, can synthesize cobalamin de novo [17], while E. coli and large part of the Vibrionaceae perform cobalamin biosynthesis only when provided with cobinamide. It is interesting to observe that the genes belonging to the de novo pathway are only shared by Archea, some other organisms like Salmonella, Pseudomonas and Vibrio MED222.

Finally cluster 6 is enriched by map2010 (ABC transporter), map2020 (two-component system), map2030 and map2031 (bacterial chemotaxis), map2040 (Flagellar assembly) and map3090 (type II secretion system). This cluster contains genes shared with a high similarity by all
Vibrio and with a lower similarity with Photobacterium profundum species. Among the Vibrio species the organisms showing the highest similarity (Tab. 3) are V. cholerae strains.

**Vibrionaceae specific genes**

We identify 1940 clusters specific to the Vibrionaceae. All the Vibrionaceae considered in the analysis share 108 clusters. Among these genes we identify ToxR and ToxS genes. ToxR gene encodes a transmembrane regulatory protein firstly identified in *V. cholerae*, in which it co-ordinates many virulence factors in response to several environmental parameters [18]. *V. cholerae* ToxR activity is enhanced by a second transmembrane protein, ToxS, encoded downstream toxR [19]. This family of proteins is involved in response to temperature, pH, osmolarity and in *Photobacterium profundum* SS9, a piezophilic bacterium, to hydrostatic pressure [20]. The widespread presence of these genes among the *Vibrionaceae* suggests their importance in regulatory mechanisms.

We identify two other noteworthy groups of genes composed by 257 and 160 genes respectively shared just by two strains, mainly annotated as "hypothetical protein". The first group of genes is shared between *Photobacterium profundum* SS9 and *Photobacterium profundum* 3TCK, while the second is shared between *V. vulnificus* CMCP6 and YJ016. These strains are closely related and this explains the high number of shared genes; while, inside the *Vibrionaceae* family, the number of specific shared genes highly decreases, showing a high inter-species variability (Fig. 6)

**Prophages and transposases**

Prophages recover different biological roles both as quantitatively important genetic elements of the bacterial chromosome, and as vectors of lateral gene transfer among bacteria, due to their characters of mobile DNA elements. Indeed, numerous virulence factors from bacterial pathogens are phage encoded. It was postulated that this role of prophages is not limited to pathogenic bacteria but some adaptations of nonpathogenic strains to their ecological niche might also be mediated by prophages acquisition [21].

To better understand the importance of mobile elements within *Vibrionaceae* family, we performed a hierarchical cluster analysis using gene profiles annotated as “phage protein” and “transposase”, for a total of 172 clusters of genes (Fig 7). We found that a high inter-strain genetic variability exists and phages and transposases are both shared by almost all *Vibrionaceae*, and specific to just some organisms. We identified five major clusters of mobile elements that are specific to a single organism. A group composed by 26 clusters containing both transposase and phage proteins seem to be unique to *V. splendidus* 12B01 (Fig 7). Another one composed by 16 clusters is specific of *V. vulnificus* CMCP6 (Fig 7) while *V. parahaemolyticus* has a cluster of 11 genes (Fig 7). Moreover there is another group of transposases and phage genes shared mainly by *V. cholerae* 0395, *Shewanella oneidensis* and *V. cholerae* V51 (Fig 7). Finally a big cluster of almost 30 genes, all predicted to code for transposases, was found in *P. profundum* SS9 genome (Fig. 7). The high presence of transposases in this bacterium seems to correlate with its deep-sea habitat, a feature presumably shared with other deep-sea microorganisms [12]. As shown in Fig 7, many of the clusters well conserved in an organism, are partially shared with a low similarity by other organisms. This agrees with the idea that prophages are not maintained in the genome over a long period of time and part of their genes may be deleted from the chromosome. Moreover, microarray analysis and PCR scanning demonstrated that prophages are frequently strain specific within a given bacterial species [22-24]. According to the modular theory of phage evolution, phage genomes are mosaics of modules, groups of genes functionally related, that are free to recombine in genetic exchanges between distinct phages infecting the same cell [21]. This can result in the occurrences of different part of phage distributed in far related genomes. Phylogenetic profile of some transposases is similar to the phage ones, suggesting a possible transfer mechanism phage-mediated for such mobile elements.

**Conclusion**

In this work we propose an improved phylogenetic profile analysis on 14 Vibrionaceae genomes, to study this family on the basis of gene content. Using a phylogenetic profile for each cluster of genes defined as the median of all the profiles belonging to the cluster (meta-profile) we investigate the evolution of groups of ORFs belonging to the entire family. A two-way cluster analysis allows us to identify similarity structures on the entire phylogenetic matrix composed by 8,239 clusters of genes and 320 organisms. The phylogenetic tree obtained with the cluster analysis does not reflect the global evolutionary tree because of the Vibrionaceae ORFs dataset used for the analysis, but rather can be considered as the Vibrionaceae perspective of bacterial diversity. The phylogenetic tree reflects the evolutionary processes that shape genomes, as lateral gene transfer, genes genesis and loss. In this context, the tree allows to group together genomes on the base of their global gene content.

We found that genomes belonging to the same taxonomic group tend to cluster together and that Vibrionaceae species are closely related. Moreover organisms belonging to the same or closely related taxa split into different subgroups, confirming the existence of a high variability among lineages, due to genetic and evolutionary process
such as lateral gene transfer, concerted evolution and genes duplication.

On the other hand several groups of genes characterised by different homogeneous profiles have been identified. In particular we found, 1) a set of conserved genes (with a high similarity values across all organisms) that reflects the “minimal genome” composition defined in other previous works; 2) a set of genes mainly shared by Vibrionaceae and other Gamma proteobacteria and 3) genes specific to different sets of Vibrionaceae.

Finally a further analysis on prophage and transposase has confirmed the high inter-strain genetic variability even among closely related species.

The increasing number of genomes included in this type of analysis surely add new sources of variability and noise, anyway we think that the use of meta-profiles can be useful for complexity reduction and data analysis to study global gene evolution.
Figure 7
Two-way hierarchical cluster analysis performed on prophage and transposase proteins. The blue bars highlight the more interesting clusters of genes such as for example the *Vibrio cholerae* CTX prophage.
Methods
Datasets
The Vibrionaceae species used in this analysis were selected among the freely available complete and draft genome sequences. The proteomes of V. cholerae N16961, V. parahaemolyticus, V. vulnificus YJ016, V. vulnificus CMCP6, V. fischeri ES114, Photobacterium profundum S99 were downloaded from the NCBI ftp site [25]. Protein sequences of Vibrio cholerae MO10, Vibrio cholerae 0395, Vibrio cholerae RC385, Vibrio cholerae V51, Vibrio cholerae V52 were downloaded from the NCBI genome database, while sequences of Vibrio MED222, Vibrio splendidus 12B01, Photobacterium profundum 3TCK from the J. Craig Venter Institute web site.

The 320 complete genomes update at 03/06 were downloaded from the NCBI ftp site.

Similarity search and phylogenetic profile construction
All the Vibrionaceae ORFs were merged generating a redundant list of 59,669 proteins and were compared to all open reading frame from 320 bacterial and archeal genomes using Blastp. To determine the presence of an orthologous we used a combination of three different thresholds; a similarity value equal to or higher than 30%, an alignment length equal or higher than 70% and an Evalue score lower than or equal to e-6. After determining the presence of an orthologous gene, we computed a similarity index I for each pair of orthologous (a point of the phylogenetic profile) as follow:

\[ I = \frac{S_{q\alpha}}{S_{qq}} \cdot \frac{\min(l_q, l_s)}{\max(l_q, l_s)} \]

where \( l_q \) and \( l_s \) are the query and subject length sequence respectively and \( S_{q\alpha} \) is the similarity score between the query and the subject sequence. \( S_{qq} \) is defined as follow:

\[ S_{qq} = \sum_{i=1}^{M} \alpha_{A_qi, A_s} + GP \]

where \( M \) is the match length between the query and subject sequence; \( A_q \) and \( A_s \) respectively the query and subject amino acid in position \( i \); \( \alpha \) the BLOSUM substitution matrix value for amino acid pair \( A_q \) and \( A_s \) and \( GP \) the gap penalty. \( GP \) is defined as follow:

\[ GP = GOP + GEP(k-1) \]

where \( GOP \) is the Gap Open Penalty set to -11, \( GEP \) the Gap Extension Penalty set to -1 and \( k \) the gap length. \( S_{qq} \) represents the score of the self-aligned query sequence.

\( S_{qq} \) is always smaller than \( S_{q\alpha} \) and the score \( S \) range between 0 and 1. In order to take into account also the different sequence lengths, we multiplied the score \( S \) by the ratio between the minimum length between query and subject and the maximum length between query and subject. In this way the total score is weighted on the base of the length, resulting in a lower similarity value if the lengths of the sequences are different.

The phylogenetic profile for each ORF is an array of index \( l \) with length equal to the number of genomes considered (320).

ORFs clustering
The redundant list of 59,669 Vibrionaceae ORFs contained multiple copies of the same genes due to the presence of conserved genes in the considered genomes. In order to reduce the redundancy, we clustered proteins using a two-step approach. The first step is based on COG (Cluster of Othologous Genes) annotation. COG classifies conserved genes according to their homologous relationships. All the Vibrionaceae ORFs were annotated using COG clusters and proteins sharing the same COG code were considered belonging to the same cluster. In particular, the annotation process consists of a similarity search of all the ORFs against the COG proteins using blast and considering the best hit for each protein. 43,024 ORFs presented a similarity with a COG entry, producing 2,463 different clusters. In the second step, the remaining 16,645 ORFs without similarity with any COG entry were clustered using CD-HIT software [26]. CD-HIT program clusters protein sequence database at high sequence identity threshold and efficiently removes high sequence redundancy. This last clustering process produced 9,613 different groups of similar proteins.

Finally from the 12,076 total clusters obtained by this methodology, those composed by ORFs that do not have any orthologous genes (with a phylogenetic profile composed by an array with all zero values except for one position match with itself) were eliminated, resulting in a dataset composed by 8,239 distinct clusters.

The final phylogenetic profile for each cluster (meta-profile) was defined as the median of all the profiles belonging to the cluster. At the end of these procedures the final phylogenetic matrix was composed by 8,239 rows (cluster of genes) and 320 columns (organisms). In each cell the median of the index in the cluster was reported.

Cluster analysis
Several clustering techniques have been used to identify the similarity structure underneath our data. A k-means and a two-way hierarchical cluster analysis with Euclidean distance and complete linkage were performed on the phylogenetic matrix.
The goal of a cluster analysis is to partition the elements into subsets without any constraints or a priori information, so that two criteria are satisfied: homogeneity, elements inside a cluster are highly similar to each other; and separation, elements from different clusters have low similarity to each other.

The Figure of Merit (FOM) is a measure of fit of the expression patterns for the clusters produced by a particular algorithm that estimates the predictive power of a clustering algorithm. It is computed by removing each sample in turn from the data set, clustering genes based on the remaining data, and calculating the fit of the withheld sample to the clustering pattern obtained from the other samples. On our data FOM analysis identified the best number of k-means clusters between 10 and 15. We decided to set k (in the k-means analysis) equal to 14. In each of these 14 clusters subsequent hierarchical cluster analysis was performed with bootstrap cluster assessment. All the previous analyses were performed with TMEV software [27], freely available at [28].

Enrichment categories

Each cluster of genes has been annotated according to COG code, GO terms and KEGG pathway maps. Class enrichment (with respect to the entire matrix) has been calculated according to the hypergeometric distribution that was used to obtain the chance probability of observing the number of genes annotated with a particular COG, GO and KEGG category among the selected cluster. The probability P of observing at least k genes of a functional category within a group of n genes is given by:

\[
P = \sum_{i=k}^{n} \binom{f}{i} \binom{g-f}{n-i} \binom{g}{n}
\]

where f is the total number of genes with the same category (in the matrix) and g is the total number of genes in our matrix.

Authors’ contributions

NV conceived of the study, performed all the bioinformatic and statistical analysis (similarity search and phylogenetic profile construction, ORFs clustering, cluster analysis and COG, KEGG and GO enrichment) and drafted the manuscript. AV and SC participated in the design and coordination of the study, revised the manuscript and participated in the interpretation of the results. CR participated in the design of the study and coordinates the statistical analysis. GV participated in the design and in the coordination of the study, in the interpretation of the results and revised the manuscript. All authors read and approved the final manuscript.

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References

1. Bushman F: Lateral DNA transfer: mechanisms and consequences. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; 2002.
2. Wagner PL, Waldor MK: Bacteriophage control of bacterial virulence. Infect Immun 2002, 70:3985-3993.
3. Pellegrini M, Marcotte EM, Thompson MJ, Eisenberg D, Yeates TO: Assigning protein functions by comparative genome analysis: Protein phylogenetic profiles. Proc Natl Acad Sci USA 1999, 96:4285-4288.
4. Enault F, Suire K, Abergel C, Poirot O, Claverie JM: Annotation of bacterial genomes using improved phylogenomic profiles. Bioinformatics 2003, 19(Suppl 1):i105-i107.
5. Sun J, Xu J, Liu Z, Liu Q, Zhao A, Shi T, Li Y: Refined phylogenetic profiles method for predicting protein-protein interactions. Bioinformatics 2005, 21:3409-3415.
6. Lawrence JG, Hendrickson H: Genome evolution in bacteria: order beneath chaos. Current Opinion in Microbiology 2005, 8:572-578.
7. Gil R, Silva FJ, Pereto J, Moya A: Determination of the core of a minimal bacterial gene set. Microbiol Mol Biol Rev 2004, 68(3):518-37. Review.
8. Moran NA, Mira A: The process of genome shrinkage in the obligate symbiont Buchnera aphidicola. Genome Biol 2001, 2(12):RESEARCH0054. Epub 2001 Nov 14
9. Motan NA: Accelerated evolution and Muller’s ratchet in endosymbiotic bacteria. Proc Natl Acad Sci USA 1993(7):2873-8. 1996 Apr 2
10. Charlebois RL, Doolittle WF: Computing prokaryotic gene ubiquity: Rescuing the core from extinction. Genome Res 2004, 14:2469-2477.
11. Vezzi A, Campanaro S, D’Angelo M, Simonato F, Vitulo N, Lauro FM, Cestaro A, Malacrida G, Simionato B, Cannata N, Romualdi C, Bartlett DH, Valle G: Life at depth: Photobacterium profundum genome sequence and expression analysis. Science 2005, 307:1459-61.
12. DeLong EF, Preston CM, Mincer T, Rich V, Hallam SJ, Frigaard NU, Martinez A, Sullivan MB, Edwards KR, Brito BR, Chisholm SW, Karl DP: Community genomics among stratified microbial assemblages in the ocean’s interior. Science 311(S760):496-503. 2006 Jan 27
13. Chen CY, Wu KM, Chang YC, Chang CH, Tsai HC, Liao TL, Liu YM, Chen HJ, Shen AB, Li JC, Su TL, Shao CP, Lee CT, Hor Li, Tsai SF: Comparative genome analysis of Vibrio vulnificus, a marine pathogen. Genome Res 2003, 13(12):2577-87.
14. Ruby EG, Urbanowski M, Campbell J, Dunn A, Faini M, Gansalus R, Lustrroh P, Lupp C, McCann J, Millikan D, Schaefer A, Stabb E, Stevens A, Visick K, Whistler C, Greenberg EP: Complete genome sequence of Vibrio flacheri: a symbiotic bacterium with pathogenic congeners. Proc Natl Acad Sci USA 102(8):3004-9. Epub 2005 Feb 9, 2005 Feb 22
15. Camberger JL, Sandkvist M: Molecular analysis of the Vibrio cholerae type II secretion ATPase EpsE. J Bacteriol 2005, 187(1):249-56.
16. Roth JR, Lawrence JG, Bobik TA: Cobalamin (coenzyme B12): synthesis and biological significance. Annu Rev Microbiol 1996, 50:137-81. Review
17. Lawrence JG, Roth JR: Evolution of coenzyme B12 synthesis among enteric bacteria: evidence for loss and reacquisition of a multigene complex. Genetics 1996, 142(1):1-24.
18. Miller VL, Taylor RK, Mekalanos JJ: Cholera toxin transcriptional activator ToxR is a transmembrane DNA binding protein. Cell 1987, 48(2):271-279. 1987 Jan 30
19. DíRia V, Mekalanos JJ: The biphasic interaction between two membrane regulatory proteins, ToxR and ToxS, results in signal transduction and transcriptional activation. Cell 1991, 64:29-37.
20. Welch TJ, Bartlett DH: Identification of a regulatory protein required for pressure-responsive gene expression in the deep-sea bacterium Photobacterium species strain SS9. Mol Microbiol 1998, 27:977-85.
21. Canchaya C, Proux C, Fournous G, Bruttin A, Brussow H: Prophage genomics. Microbiol Mol Biol Rev 2003, 67:238-76.
22. Murray AE, Lies D, Li G, Nealson K, Zhou J, Tiedje JM: DNA/DNA hybridization to microarrays reveals gene-specific differences between closely related microbial genomes. Proc Natl Acad Sci USA 98(17):9853-8. Epub 2001 Aug 7. 2001 Aug 14

23. Chan K, Baker S, Kim CC, Detweiler CS, Dougan G, Falkow S: Genomic comparison of Salmonella enterica serovars and Salmonella bongori by use of an S. enterica serovar typhimurium DNA microarray. J Bacteriol 2003, 185(2):553-63.

24. Ohnishi M, Terajima J, Kurokawa K, Nakayama K, Murata T, Tamura K, Ogura Y, Watanabe H, Hayashi T: Genomic diversity of enterohemorrhagic Escherichia coli O157 revealed by whole genome PCR scanning. Proc Natl Acad Sci USA 99(26):17043-8. Epub 2002 Dec 12. 2002 Dec 24

25. NCBI ftp site. ftp://ftp.ncbi.nih.gov/genomes/Bacteria

26. Li W, Jaroszewski L, Godzik A: Tolerating some redundancy significantly speeds up clustering of large protein databases. Bioinformatics 2002, 18:77-82.

27. Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Clark M, Currier T, Thiagarajan M, Sturn A, Snuffin M, Rezantsev A, Popov D, Rytsov A, Kostukovich E, Borisovsky I, Liu Z, Vinsavich A, Trush V, Quackenbush J. TM4: a free, open-source system for microarray data management and analysis. Biotechniques 2003, 34:374-8.

28. TMEV web site [http://www.tm4.org/index.html]