Proteinortho:
Detection of (Co-)Orthologs in Large-Scale Analysis

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Abstract

Background: Orthology analysis is an important part of data analysis in many areas of bioinformatics such as comparative genomics and molecular phylogenetics. The ever-increasing flood of sequence data, and hence the rapidly increasing number of genomes that can be compared simultaneously, calls for efficient software tools as brute-force approaches with quadratic memory requirements become infeasible in practise. The rapid pace at which new data become available, furthermore, makes it desirable to compute genome-wide orthology relations for a given data set rather than relying on relations listed in databases.

Results: The program Proteinortho described here is a stand-alone tool that is geared towards large data sets and makes use of distributed computing techniques when run on multi-core hardware. It implements an extended version of the reciprocal best blast hit method. We apply Proteinortho to compute orthologous proteins in the complete set of all 711 eubacterial genomes available at NCBI at the beginning of 2009. We identified thirty proteins present in 99% of all bacterial proteomes.

Conclusions: Proteinortho significantly reduces the required amount of memory for orthology analysis compared to existing tools, allowing such computations to be performed on off-the-shelf hardware.

Availability: The source code of Proteinortho can be obtained under the GPLv2 (or later) from http://www.bioinf.uni-leipzig.de/Software/proteinortho/
Background

Genome annotation largely depends on the determination of sequence intervals that are homologous, and if possible, orthologous to sequences of known identity and function in related genomes. Orthologous genes (orthologs) are derived from a common ancestor by a speciation event [1]. Orthologs are of particular interest because they can be expected to have maintain at least part of their (ancestral) biological function. For protein-coding genes, several well-known databases, including InParanoid [2], OrthoMCL-DB [3], COG [4], Homogene [5], eggNOG [6], OMA Browser [7] and Ensembl Compara [8] compile such information. Their content is restricted to data previously published in comprehensive databases of protein sequences such as UniProt [9]. Updates with additional proteomic data thus are published relatively infrequently. The modern high-throughput technologies, however, produce huge amounts of protein data and even larger amounts of transcript data that are computationally translated to putative polypeptide sequences. Oftentimes, therefore, it would be desirable to generate the orthology relation for a particular data set, so that the availability of orthology data does not limit the set of species or genes that can be included.

The computation of genome-wide orthology data, however, is a challenging and time consuming task with the currently available tools. In many cases, orthologs cannot be identified unambiguously by means of sequence comparison. The main difficulty arises from the presence of paralogs (homologous genes within the same genome) which can make it very difficult to recognize the correct ortholog among the other homologs. Gene duplications in following the speciation, furthermore, create two or more genes in one lineage that are, collectively, orthologous to one or more genes in another lineage. Such genes are known as co-orthologs [10].

The most widely used approach to identify (putative) orthologs between two species is the reciprocal best blast hit method [11–15]. It was more recently extended e.g. in OrthoMCL [16] and MultiParanoid [17] to detect (co-)orthologs within multiple species. All these tools, however, are limited to relatively small sets of species. In practise, analyzing the complete proteomes of more than about 50 procaryote species goes beyond the capabilities of standard hardware and requires access to supercomputer resources. The crucial limitation is the amount of memory required to hold results of all pairwise comparisons. In contrast, Proteinortho can deal with hundreds of species together containing millions of proteins by processing the output of blast comparisons already on the fly.

Results and Discussion

Orthology prediction

Theory

As for other approaches to large-scale orthology detection, the starting point is a complete collection of pairwise comparisons, typically performed using blast. For simplicity of presentation, we assume that the individual sequences that are compared represent proteins, although algorithms and pipelines are applicable also to other sequence data such as non-coding RNA genes or conserved DNA regions.

Typically, the results of pairwise comparisons are ranked by similarity, for instance based on blast statistics, evolutionary distances, or genome rearrangement analysis [7, 16, 18]. High-ranking hits across multiple species then have to be combined in order to determine orthologous groups. However, these groups usually do not readily provide detailed insights since they can contain large numbers of related genes for each species. Hence, meaningful units have to be identified. For this purpose, a variety of clustering algorithms have been employed, as in the MCL-algorithm [16, 19, 20]. Alternatively, data were curated by manual postprocessing [4].

We will argue here that orthology determination can be understood as the problem of finding nearly disjoint maximal nearly-complete multipartite subgraphs in an edge-weighted directed graph $\bar{\mathcal{T}}$ whose vertices are the proteins in the input set, and whose edges connect certain pairs of similar proteins of different species. The edge weights $\omega_{x\rightarrow y}$ encode the similarity of $x$ and $y$. In our implementation, the bit scores of the blast hit ($x \rightarrow y$) will serve as edge weight. An $E$-value cut-off is used beyond which blast hits are not included into $\bar{\mathcal{T}}$.

In order to motivate our point of view, we first consider an idealized data set (see Fig. 1) in which (1) each protein $x$ (from species $A$) has at most one ortholog in any other species $B \neq A$. (2) if $y \in B$ is an ortholog of $x \in A$, then a blast search of $x$ against $B$ yields at least one hit and (3) the unique best hit of query $x$ against $B$ is the true ortholog $y$ of $x$. In this case, the well-known “reciprocal best hit heuristic” (RBHH) can be used to retrieve the
correct ortholog set. To see this, we construct a subgraph \( \tilde{\Upsilon}_{RBHH} \) of \( \Upsilon \) as follows: For each protein \( x \) in species \( A \) and a given species \( B \neq A \) we retain only the arc with maximal weight:

\[
(x \rightarrow y) \in \tilde{\Upsilon}_{RBHH} \text{ iff } \omega_{x \rightarrow y} = \max_{y' \in B} \omega_{x \rightarrow y'} \quad (1)
\]

The symmetric subgraph of \( \tilde{\Upsilon}_{RBHH} \), containing only reciprocal best hits, can be regarded as an undirected graph \( \Upsilon_{RBHH} \). By construction, any two vertices are connected by edges in \( \Upsilon_{RBHH} \) if and only if they are orthologs. A set of orthologs therefore corresponds to a complete multipartite subgraph of \( \Upsilon_{RBHH} \) in which every species is represented at most once. Furthermore, we note that these subgraphs are disjoint, i.e., ortholog sets correspond to the connected components of \( \Upsilon_{RBHH} \).

When applied to real data, however, RBHH usually gives rise to several artifacts. In general, therefore, it does not produce correct and complete sets of orthologs. First, gene duplications produce co-orthologs, destroying the uniqueness of best blast hits. The blast comparison of two species both containing two co-orthologs will in general produce slightly different scores among those genes, so that \( \tilde{\Upsilon}_{RBHH} \) in general will not contain all arcs between them, see Fig. 2A. RBHH now extracts the symmetric part of \( \tilde{\Upsilon}_{RBHH} \), i.e., it removes all non-reciprocal edges. Thus the undirected graph \( \Upsilon_{RBHH} \) has an edge \( \{x, y\} \) if and only if both \( (x, y) \) and \( (y, x) \) are arcs in \( \tilde{\Upsilon}_{RBHH} \). A missing arc in the example of Fig. 2A thus translates into a missing edge in \( \Upsilon_{RBHH} \). Ortholog sets, therefore, are still located in disjoint connected components, but they are only approximately multipartite and there is no guarantee that they remain connected. As a remedy, it could be proposed to use the \( k \)-best blast hits instead of a single best one. This leads, however, to another type of artifact: If highly diverged homologs or spurious blast hits are present, Fig. 2B, edges between non-orthologous proteins might be included.

In order to reduce such problems, we suggest to introduce a similarity cut-off value that itself depends on the quality of the matches, i.e., to consider all those blast hits of each query against a given species whose \( E \)-values are only slightly smaller (by a factor \( f < 1 \) ) than the queries’ best blast hit. More precisely,

\[
(x \rightarrow y) \in \tilde{\Upsilon}^* \text{ iff } \omega_{x \rightarrow y} \geq f \max_{y' \in B} \omega_{x \rightarrow y'} \quad (2)
\]

The symmetric part \( \Upsilon^* \) of \( \tilde{\Upsilon}^* \) now retains more edges than \( \Upsilon_{RBHH} \). In particular, it includes all the edges connecting similar co-orthologs. On the other hand, the threshold at fairly high bit scores disconnects at least most of the more distant homologs. Sets of (co-)orthologs thus appear in \( \Upsilon^* \) as nearly complete multipartite subgraphs. Typically they will contain more than one node from the same species, among them, in particular, all in-paralogs. Although this approach strongly reduces the problem with spurious edges, \( \Upsilon^* \) may also contain additional edges connecting two or more sets of (co-)orthologs.

The problem of finding maximal complete multipartite subgraphs of a graph is NP complete [21]. Furthermore, we have seen above that \( \Upsilon^* \) may lack a few edges that should connect orthologs, while at the same time there are also some additional “false positive” edges. Hence, orthology detection might be formulated as an editing problem, asking for a decomposition of \( \Upsilon^* \) into a disjoint collection of complete multipartite subgraphs with the minimal number of edge insertions and deletions.

Since no efficient approaches to this combinatorial optimization problem seem to be known, it appears fruitful to resort to a heuristic approach that employs a somewhat different point of view: nearly complete multipartite subgraphs are very dense subgraphs, which in our case either form connected components on their own, or which are connected to other dense clusters by a few additional edges.
Figure 2: Reciprocal best hit heuristic (RBHH).

(A) If there is a pair of divergent co-orthologs \( x_1, x'_1 \), and \( y_1 \) and \( y'_1 \), resp., it is possible that there are no reciprocal best blast hits. In this situation, RBHH will not identify any orthologs. (B) One possible remedy is to include the second best blast hits \( (n=2) \). However, in this case highly similar orthologs \( x_2 \) and \( y_2 \) as well as \( x_3 \) and \( y_3 \), which in principle can clearly be divided, can get combined. (C) ProteinOrtho uses an adaptive approach that is (1) flexible with respect to the number of more diverged orthologs in absence of a reciprocal best blast hit and (2) will not intermix ortholog-groups that can be disentangled easily because of large differences in pairwise similarity.

The problem thus is to determine for each connected component of \( \mathcal{Y}^* \) whether it is sufficiently densely connected, and if not, to partition it into its densely connected components by removing the spurious edges connecting them. Here, we approach this issue by means spectral partitioning [22].

**Implementation**

**blast searches.** ProteinOrtho expects fasta files containing either nucleic acid or amino acid sequences as input. In contrast to previous approaches, however, ProteinOrtho does not build a large database containing all proteins but rather keeps the protein complements of different species separate. This has multiple advantages: (1) the blast step can be partitioned into multiple runs. The available processor cores are used efficiently as each blast process can utilize one processor core to the full, which is usually not the case if threading is handled by blast itself. Furthermore, the blast jobs can be distributed to several computers in a network. (2) The computationally expensive blast search of species against themselves (which is implicit in searching a comprehensive protein database) can be avoided. (3) The \( E \)-values returned by blast depend only on the proteome of each input species, not on the database of all input sequences. (4) The scope of the analysis can be extended without the need to re-compute blast comparisons that have already been computed earlier. The \( E \)-values remain unchanged when species are included or excluded from the analysis. (5) Compared to the strategy of OrthoMCL, the \( E \)-values for comparisons between distant species are smaller while the \( E \)-values of related species are larger. Therefore, ProteinOrtho implicitly enforces more exact hits for related proteomes but allows less exact hits for larger evolutionary distances.

ProteinOrtho can use several PCs with a shared storage (such as an NFS file system). The implementation of distributed computing is illustrated in Additional File 3. Furthermore, cluster infrastructure (e.g. MPI or SGE) is supported. A benchmark illustrating the performance improvements is shown in Figure 3a. ProteinOrtho is faster than OrthoMCL even if only a single processor core is available.

Most importantly, the algorithm outlined in the previous section avoids the memory bottleneck that limits previous approaches. Suppose our input set comprises \( N \) species with, on average, \( m \) genes. The size of the input is thus \( n = N \times m \) proteins. Instead of storing all \( n \times n \) pairwise blast scores, ProteinOrtho processes the comparisons between any two species \( A \) and \( B \) immediately: first the blast hits are filtered by two additional criteria: (1) The alignment must exhibit a minimum level of sequence identity. (2) The alignment must cover at least a minimum fraction of the query protein. This second rule ensures that fusion genes such as rice OsUK/UPRT1 [23] are eventually assigned as homologs of the dominating part of the protein. Then equ.(2) is evaluated for all \( x \) in \( A \), so that ProteinOrtho directly constructs the
Figure 3: CPU time and memory requirements of Proteinortho. (a) The speed benchmark was performed using an E. coli strain with 4132 proteins on an eight core Intel Xeon system using one thread (1) and eight threads (8) at 2.33GHz. The encoded proteins were used multiple times to simulate multiple (identical) species. This is the worst case scenario for Proteinortho since in this case every protein has a link to at least one protein in every other species. Proteinortho is significantly faster than OrthoMCL. Using multiple threads we observe a substantial speed up.

(b) The memory benchmark is performed using the same set as in (a). OrthoMCL quickly exhausts memory for larger sets. Proteinortho clearly performs more efficient, even though this artificial scenario is a more complex case than real world analysis. Both benchmarks outline that Proteinortho allows comprehensive studies which were not possible before.

Spectral partitioning. First we reduce the problem by determining the connected components of $\tilde{\mathcal{T}}^*$ since these can be treated separately. We use the well-known breath-first search approach [24] to this end. In order to check whether a connected component $\Xi$ is sufficiently dense to represent a single set of (co-)orthologs of a gene in a single species. The value of $a$ is independent of the size of the data set. Empirically, we found $a \leq 1$ in all datasets investigated so far. Thus Proteinortho saves a factor $n^2/N^2ma = m/a \geq m$ of memory. Note that prokaryotes have $m \approx 10^3 \ldots 10^4$ proteins.

The “Fiedler vector” $x_2$, i.e., the eigenvector of $L$ to eigenvalue $\alpha_2$ can be used to find a partition of $\Xi$ into two connected components, one consisting of the vertices for which $x_2$ has positive entries and one for which $x_2$ has negative entries [27]. This decomposition is iterated until $\Xi$ is partitioned into com-
ponents with algebraic connectivity $\alpha^2 > 0.1$, minimum 50% identity, and 50% coverage of query sequence, similarity threshold $f = 0.95$) Both Proteinortho and OrthoMCL report fewer groups than listed in the COG database. This is not surprising, however, since COG contains multiple co-orthologs while both stand-alone tools apply clustering algorithms to avoid such assignments.

Fig. 5 presents the outcome in more detail. Proteinortho and OrthoMCL report comparable results. Proteinortho is more stringent. The amount of completely new groups which have to be regarded as false positives is considerably lower.

### Evaluation of Proteinortho

We compared Proteinortho with the COG database [4] and OrthoMCL [3]. The latter is the main competitor in terms of speed and memory. The COG database provides a manually curated data set that can be regarded as more reliable than fully automated approaches. For benchmark analysis, a set of 16 randomly chosen bacteria from three different classes (six Gram-positive bacilli, six gamma- and four alpha-proteobacteria) are used. The input set comprises 53,623 protein sequences.

Fig. 4 summarizes the size distribution of co-ortholog sets for the three approaches. Using default settings ($E$-value cutoff $10^{-10}$, algebraic connectivity threshold $\alpha^2 > 0.1$, minimum 25% identity, 50% coverage of query sequence, similarity threshold $f = 0.95$) both Proteinortho and OrthoMCL report fewer groups than listed in the COG database. This is not surprising, however, since COG contains multiple co-orthologs while both stand-alone tools apply clustering algorithms to avoid such assignments.

Fig. 5 presents the outcome in more detail. Proteinortho and OrthoMCL report comparable results. Proteinortho is more stringent. The amount of completely new groups which have to be regarded as false positives is considerably lower. OrthoMCL reports slightly more groups. Even though both tools were applied with the same E-value threshold the results vary due to the different blast strategies. Most pairwise E-values derived by Proteinortho tend to be somewhat larger than those derived by OrthoMCL for the same set of proteins. In turn, OrthoMCL reports some more and larger groups. However, the overall outcome can be regarded as similar while the runtime and memory requirements of Proteinortho are largely reduced in comparison to OrthoMCL.

### Domain-wide commons

In order to demonstrate that Proteinortho is suitable for large-scale analyses we asked Which proteins can be found in all bacterial species? Proteins that are conserved domain-wide are likely to be useful for the construction of a phylogeny of eu- bacteria as an alternative to the prevalent usage of 16S rRNA sequences [28]. They can also serve as protein-based markers for identifying novel bacterial species as members of an established phylogenetic group. In addition, they can give insight into basic protein equipment of bacterial life. Hence, we applied Proteinortho to the set of all cubacterial proteomes available at NCBI in late 2009.

The input dataset comprises 2,155,620 proteins annotated in 711 bacterial genomes. The Proteinortho run took less than two weeks using 50 processor cores (Intel Xeon at 2.00-2.33GHz) distributed over multiple PCs. Only 2 GB memory were required. OrthoMCL could not be employed for this task on the hardware available in our lab. Extrapolating from the benchmarks in Fig. 3, we estimate that hundreds of gigabytes of memory and years of runtime would have been required.

Proteinortho identified thirty proteins as core of the bacterial protein complement, occurring in 99% of all 711 free-living and endosymbiotic bacteria:

- elongation factor Tu (often co-orthologous to elongation factor 1-alpha)
- elongation factor G
- translation initiation factor IF-2
- RNA polymerase subunit $\beta$ and $\beta'$
- ATP-dependent metalloprotease FtsH
- O-sialoglycoprotein endopeptidase
- methionine aminopeptidase
- F0F1 ATP synthase subunit $\alpha$ and $\beta$'s
- dimethyladenosine transferase
- 1 ribosomal protein of the $30S$ rRNA subunit
- 3 ribosomal proteins of the $50S$ rRNA subunit
- 3 GTP-binding proteins
- 12 tRNA synthetase

About one third of these proteins could not be found in the two species with the smallest proteomes in our data set: Candidatus Carsonella ruddii PV and Candidatus Sulcia muelleri GWSS. Both are
endosymbionts that are considered as organelle-like [29,30]. Numerous genes that are otherwise considered to be essential for life have been reported as missing in both species. A more detailed and larger list of domain-wide common proteins can be downloaded at http://bioinf.pharmazie.uni-marburg.de/supplements/proteinortho/.

Conclusions

Proteinortho implements a blast-based approach to determine sets of (co-)orthologous proteins or nucleic acid sequences that generalizes the best reciprocal blast hit heuristic. The software is optimized for large data sets, and in particular provides a drastic reduction of the memory requirements compared to earlier tools. It can therefore be run on off-the-shelf PC hardware for large data sets. Our implementation scales very well with the number of available processor cores. The blast searches can be trivially parallelized and distributed easily to multiple PCs without the need for a cluster management system, while deployment to existing cluster infrastructure is also supported.

Proteinortho views orthology detection as a variant of graph clustering since co-orthologous sets correspond to maximal complete multipartite subgraphs, which at the same time are well separated

Figure 4: Comparison of the results of Proteinortho with different thresholds of the normalized algebraic connectivity $\alpha_2^* = 0...0.8$ with the COG-database and OrthoMCL for a dataset consisting of 16 randomly chosen bacterial proteomes. The vertical dashed line marks the transition from clusters containing mainly a single ortholog from each species to sets including co-orthologs. The COG-database reports many large groups which often include co-orthologous proteins. OrthoMCL and Proteinortho focus on highly connected subsets in order to find orthologous sets and thus split those groups. Thereby, Proteinortho’s clustering algorithm becomes more stringent with increasing values of $\alpha_2^*$ in splitting in particular large groups. While these groups are left intact for $\alpha_2^* = 0$, thresholds of 0.5 and higher drastically reduce the fraction of included co-orthologs.

Figure 5: Comparison of OrthoMCL and Proteinortho to the COG-database. The following assignments were defined: identity: the group equals a COG-group; subset: the group is subset of a COG-group, at least two proteins are equal; superset: the group is a superset of a COG-group, at least two proteins are equal; new: none of the above-noted criteria matched. Both tools reveal comparable results with respect to the manually curated COG-database. OrthoMCL covers more identical and differently composed groups while Proteinortho is more restrictive and reports fewer new groups which are not present in the COG-database. All groups with less than six species were omitted from the OrthoMCL and Proteinortho data. See Additional File 3 for comparisons with different minimal coverage.
from each other. Due to the unavoidable noise in the real data, however, co-orthologous sets appear as dense subgraphs without clearly recognizable low-weight cuts. This property is measured quite well by the algebraic connectivity. At the same time, low-weight cuts between dense regions are identified very well by the corresponding Fiedler vector. We therefore employ spectral partitioning instead of a direct graph clustering approach. The quality of the co-orthologous sets proposed by Proteinortho is comparable to the performance of OrthoMCL.

Both time and memory requirements are significantly reduced compared to earlier approaches, enabling applications that were infeasible before. For instance, we applied Proteinortho to the complete set of 2.1 million proteins from the 711 bacterial genomes available at NCBI at the beginning of 2009. We found 30 proteins that are present in more than 99% of the investigated sequences.

Methods
All analysis with Proteinortho and OrthoMCL were applied using default values unless described otherwise. These are $E-value < 10^{-10}$, 25% percent identity, Markov Inflation Index of 1.5 for OrthoMCL and $E-value < 10^{-10}$, 25% percent identity, adaptive best hits similarity of $f = 0.95$, algebraic connectivity $> 0.1$ for Proteinortho. OrthoMCL version 1.4 was downloaded from http://orthomcl.org/common/downloads/.

Speed and memory benchmark were performed multiple times using the proteome of Escherichia coli K12 substr. MG1655 data from the NCBI. The protein ids were renamed systematically to prevent duplicated ids for benchmarking purposes which cannot be handled by Proteinortho. A script continuously observed the memory consumption and reported the maximum peak for each run, Fig. 3.

For the domain-wide commons we applied Proteinortho with default values of the parameters. Bacterial proteomes and genomes were downloaded from NCBI http://ftp.ncbi.nih.gov/genomes/Bacteria/ in March 2009. A detailed list can be found in Additional File 2. In order to recover missing annotation, we selected orthologous groups covering at least 75% of all species that are good candidates of domain-wide commons. The unique set of sequences of each orthologous group was blasted against all genomes that lack an annotated ortholog using tblastn ($E$-value $< 10^{-20}$). The best hit was then added to the orthologous group.

For evaluation we used the proteome data from the COG-database (ftp://ftp.ncbi.nih.gov/pub/COG/COG/) downloaded in November 2009. We have chosen Bacillus halodurans, Bacillus subtilis, Lactococcus lactis, Listeria innocua, Streptococcus pneumoniae TIGR4, Streptococcus pyogenes M1 GAS from the Gram-positive bacilli class, Buchnera sp. APS, Escherichia coli K12, Pasteurella multocida, Salmonella typhimurium LT2, Vibrio cholerae, Yersinia pestis from the gamma proteobacteria class and Brucella melitensis, Caulobacter vibrioides, Mesorhizobium loti, Rickettsia prowazekii from the alpha proteobacteria class. Both, Proteinortho and OrthoMCL were applied to this set. All groups with proteins covering at least 6 species were compared to the COG-database, illustrated in Fig. 4 and Fig. 5.

Authors contributions
SJP implemented an initial prototype. ML implemented, optimized, and evaluated the later versions of the program. All authors contributed to the details of implementation, testing and interpretation of the data, and collaborated in writing and approved the final manuscript.

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Additional files
Additional File 1. Algebraic connectivity and Fiedler vector.
Additional File 2. Species list for domain wide commons.
Additional File 3. Supplemental figures.