Assignment of Orthologous Genes by Utilization of Multiple Databases
The Orthology Package in R

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Abstract: The assignment of orthologous genes between species is a key issue when multiple-species approaches are conducted. This has become even more relevant over the past years, triggered by the development of high-throughput genome sequencing technologies, which enable access to complete genomes in a rapid and cost-effective way. In this paper, we present a new software that allows the user to access orthology relationships across multiple species in an easy, fast, and flexible manner. The tool collects data from three prominent freely available databases, and presents it to the user in a convenient, easily accessible way. Once the package is installed, the software works on the local computer, therewith circumventing runtime delay caused by network traffic often being a critical performance bottleneck when large datasets are studied or many organisms are investigated simultaneously. By the consequent internal usage of unique identifiers, the software disburdens the user from problems connected with the existence of synonyms or ambiguous gene denotations, a problem that often hampers a clear-cut assignment of orthologs. The software is able to display frequently occurring, complicated many-to-many orthology relationships in a visual manner. It is written in the R programming language and freely available.

1 INTRODUCTION

Multiple-species approaches become more and more important when universal, cross-species biological principles are to be investigated, for example in systems biology, cancer- or age research. Furthermore, ethical or practical reasons - *e.g.* a limited population frequency - make the usage of model organisms in biological experiments often unavoidable, and conclusions of general relevance have to be derived starting from the results obtained for the model organisms. It appears self-evident that the accurate determination of orthologs - genes with a shared ancestor separated by a speciation event - adopts a key role when using such approaches. However, a researcher aiming at carrying out an orthology search is frequently confronted with a number of problems.

First of all, by committing oneself to a single database only, important orthology relationships might be missed because of the incompleteness of present-day databases. Secondly, the search for orthologous genes is often hampered - if not made impossible - by the existence of a multitude of synonyms or other ambiguous denotations that exist for the vast majority of annotated genes. Thirdly, if web-based databases have to be used, access can be slow if large datasets are investigated or many organisms are studied at the same time. Last but not least, orthology relationships are often not in a 1-to-1 manner, so that graphical presentations of many-to-many relationships are desirable, while simple gene lists are inconvenient and hard to perceive.

Up to now, various methods for the identification of orthologous genes between species have been developed, starting from simple blast searches aiming at the identification of bi-directional hits between the genes of two species, up to advanced clustering or tree-based methods (Kuzniar et al., 2008; Kristensen et al., 2011). The results of this work have been stored in a number of publicly available databases, providing orthology information covering a growing number of species to the research community. In this paper, we utilize three prominent and comprehensive databases that have been established in order to identify orthologous genes: i) the HomoloGene database maintained at the National Center for Biotechnology Information - NCBI (Geer et al., 2010), ii) the Ensembl Compara database driven by the Ensembl project (Vilella

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Table 1: The main commands of the R orthology package and their function.

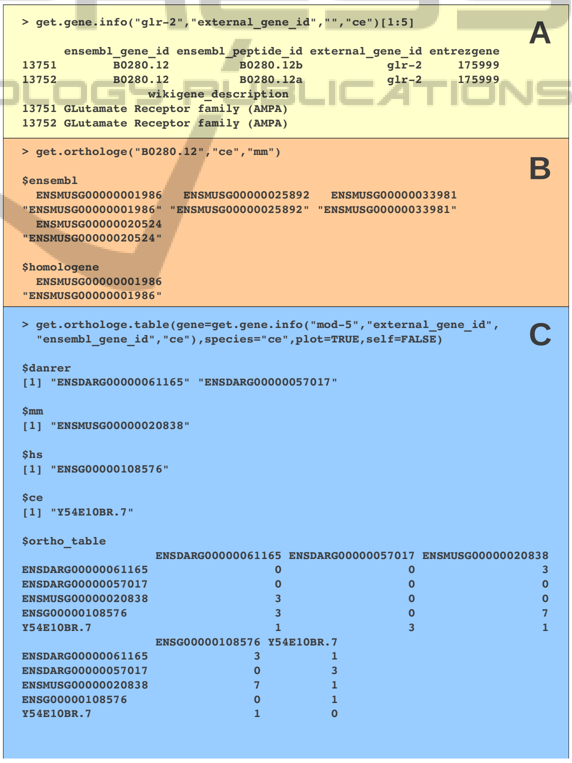
Subroutine	Task
`get.gene.info`	access different identifiers and descriptive information for genes
`get.orthologe`	get the orthologs in other species for a given gene and a given species
`is.orthologe`	test if two genes from different species are orthologous
`get.ortholog.table`	visualize orthology relationships between two or more species and return table (see figure 1)
`get.orthologe.set`	return a set of all orthologous genes between the input species

et al., 2009), and iii) the Inparanoid database held at the University of Stockholm, Sweden (Ostlund et al., 2010).

The HomoloGene database at NCBI is a system for the automated detection of homologs among the annotated genes of several completely sequenced eukaryotic genomes. The approach used to establish this database relies on clustering the input sequences from the different species based on sequence similarity on the protein level, using the blastp protein sequence alignment tool (Altschul et al., 1990). As a key feature, database creation considers taxonomy by matching sequences from closely related species first, subsequently adding sequences originating from organisms with bigger evolutionary distances to a tree constructed during this procedure (Wheeler et al., 2006). The HomoloGene database currently includes 21 species (Release 66).

The Ensembl Compara database is driven by the Ensembl project. In order to establish this database, orthology was predicted by gene tree generation using the TreeFam methodology (Ruan et al., 2008). This involves building a graph of protein similarity utilizing blastp, extracting clusters from this graph, and generating multiple alignments within each cluster. Based on these alignments, a gene tree is built and reconciled with the taxonomy tree (Vilella et al., 2009). An important feature of this approach is that sequence similarity is connected with information originating from the phylogenetic tree of the species. The respective data can be accessed using a Perl Application Programme Interface (Perl API) or the BioMart portal (Haider et al., 2009).

As a third source of orthology information, we utilize the Inparanoid database. Here, pairwise similarity scores between complete proteomes were calculated in order to construct orthology groups, seeded by the reciprocally best-matching pair. So far, this database contains 100 eukaryotic species. While InParanoid essentially relies on pairwise ortholog relationships, both HomoloGene and Ensembl Compara use heuristic as well as phylogenetic methods to infer orthologs, even though with considerably different methods. The use of multiple databases - with varying approaches to infer orthology - increases the number of identifiable orthology relationships between genes of different species. Furthermore, by using multiple databases, it becomes possible to confine oneself to robust predictions of orthology by merely considering data that are confirmed by more than one database because biases in one database can be expected to be overcome by another.



```
> get.gene.info("glr-2","external_gene_id","","ce")[1:5]

      ensembl_gene_id ensembl_peptide_id external_gene_id entrezgene
13751        B0280.12          B0280.12b            glr-2     175999
13752        B0280.12          B0280.12a            glr-2     175999
                   wikigene_description
13751 GLutamate Receptor family (AMPA)
13752 GLutamate Receptor family (AMPA)
```

```
> get.orthologe("B0280.12","ce","mm")

$ensembl
  ENSMUSG00000001986   ENSMUSG00000025892   ENSMUSG00000033981
"ENSMUSG00000001986" "ENSMUSG00000025892" "ENSMUSG00000033981"
  ENSMUSG00000020524
"ENSMUSG00000020524"

$homologene
  ENSMUSG00000001986
"ENSMUSG00000001986"
```

```
> get.orthologe.table(gene=get.gene.info("mod-5","external_gene_id",
  "ensembl_gene_id","ce"),species="ce",plot=TRUE,self=FALSE)

$danrer
[1] "ENSDARG00000061165" "ENSDARG00000057017"

$mm
[1] "ENSMUSG00000020838"

$hs
[1] "ENSG00000108576"

$ce
[1] "Y54E10BR.7"

$ortho_table
                   ENSDARG00000061165 ENSDARG00000057017 ENSMUSG00000020838
ENSDARG00000061165                  0                  0                  3
ENSDARG00000057017                  0                  0                  0
ENSMUSG00000020838                  3                  0                  0
ENSG00000108576                     3                  0                  7
Y54E10BR.7                          1                  3                  1
                   ENSG00000108576 Y54E10BR.7
ENSDARG00000061165               3          1
ENSDARG00000057017               0          3
ENSMUSG00000020838               7          1
ENSG00000108576                  0          1
Y54E10BR.7                       1          0
```

Figure 1: Usage of the main commands of the package and example output. A: Accessing different gene IDs for gene symbol glr-2 (*C. elegans*). B: Retrieval of orthologs in mouse for the selected Ensembl gene ID. In this case only Ensembl and HomoloGene provide orthologs. C: Listing of all orthologs for the mod-5 gene (*C. elegans*) in human, mouse and zebrafish. The number in the orthology table defines the source of information. If the parameter `plot` is set to `TRUE`, a network plot will be drawn (see Figure 2).

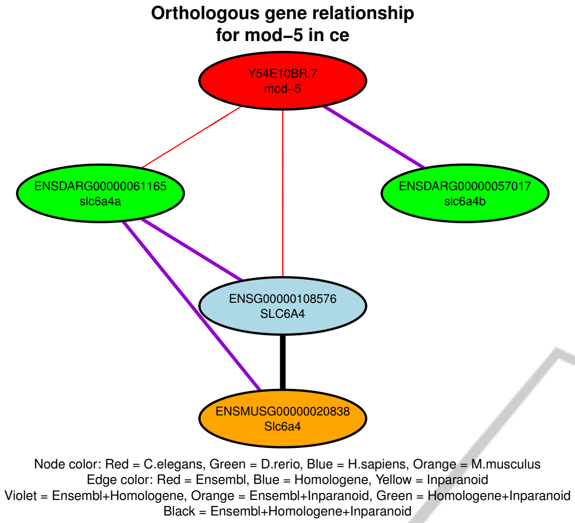

Orthologous gene relationship
for mod−5 in ce
Y54E10BR.7
mod−5
ENSDARG00000061165
slc6a4a
ENSDARG00000057017
slc6a4b
ENSG00000108576
SLC6A4
ENSMUSG00000020838
Slc6a4
Node color: Red = C.elegans, Green = D.rerio, Blue = H.sapiens, Orange = M.musculus
Edge color: Red = Ensembl, Blue = Homologene, Yellow = Inparanoid
Violet = Ensembl+Homologene, Orange = Ensembl+Inparanoid, Green = Homologene+Inparanoid
Black = Ensembl+Homologene+Inparanoid

Figure 2: Graphical representation of the orthology table produced using the command stated in figure 1 C. Here the orthology relationship is less complex, with only four orthologous genes in three other species. The orthologous connection of SLC6A5 (human) and Slc6a4 (mouse) is covered by all three databases (thick black edge), which is represented by a binary 111 (decimal 7) in figure 1 C). Self-edges are not included.

2 RESULTS

The software described in this paper allows the user to access information stored in several orthology databases with one interface only, with the advantage that the user does not have to know how the individual databases are to be operated. The software provides a collection of features which, amongst other things, allow the translation of gene names to gene identifiers, the accession of functional gene descriptions, and the retrieval of orthology relationships between two or more organisms. A few of the subroutines of the software package and their respective functions are listed in table 1.

2.1 Example Commands

The command `get.orthologe` takes a gene identifier or gene symbol as input, and outputs the orthologs in the organisms specified by the user. Upon request, all three databases listed in the introduction can be taken into account, or a subset of them.

Orthology relationships often are complicated when more than two species are involved. In this case, a graphical representation can help to understand this complexity. The latter can be accomplished using the function `get.ortholog.table`. This function displays orthology relationships in tabular form (figure 1) and visualizes these relationships in form of a network graph.

In the table, gene names of two or more organisms serve as column and row headers. Numbers in the cells of the table encode the databases that report orthology between the corresponding genes. We use binary numbers for encoding, where 1 (2^0) represents the Ensembl database, 2 (2^1) represents the HomoloGene database, and 4 (2^2) stands for an orthology relationship reported by the Inparanoid database. The actual number shown in the table is then the sum of the numbers representing the individual databases. For example, 3 in a cell of the table means that orthology of the corresponding genes is reported by the Ensembl database as well as by the HomoloGene database. Certainly, every gene is orthologous to itself. Therefore, by default, self hits are labeled 0 in the table and are suppressed in the graphical representation. However, if the parameters `plot` and `self` are set `TRUE`, the self-hits appear as 10 in the table, and self-edges are added to the plot.

Figure 2 shows a relatively simple example for a graphical representation of orthology relationships between genes, while figure 3 displays a more complicated graph. It displays the glr-2 gene in *C. elegans* and its orthologs in three other vertebrate species. In this plots, nodes define genes and edges connect orthologous genes across species. While the node color indicates species, the color of the edges stands for the database the orthology is derived from. In this case, like in many others, there is no simple 1-to-1 orthology relationship between the genes but there exists a multitude of (putative) orthologs in the organisms considered, covered by different databases (1-to-many and many-to-many).

An issue in orthology identification is if and how the order of input affects the results. For example, if the orthology request shown in figure 2 started from the human SLC6A4 gene (marked blue in the figure), the slc6a4b *D. rerio* gene (marked green, to the right) would not appear in the resulting plot, since this gene is solely listed as ortholog of the mod-5 *C. elegans* gene. In order to overcome this discrepancy, a special parameter `loop` - with default 0 - can be set to a positive integer value `n`. This causes the tool to carry out `n` iterative loops of orthology searches, in which every loop uses the results of the previous one as input.

2.2 Applications

The orthology package described here has been successfully applied in the framework of the JenAge project, a collaboration between several institutions

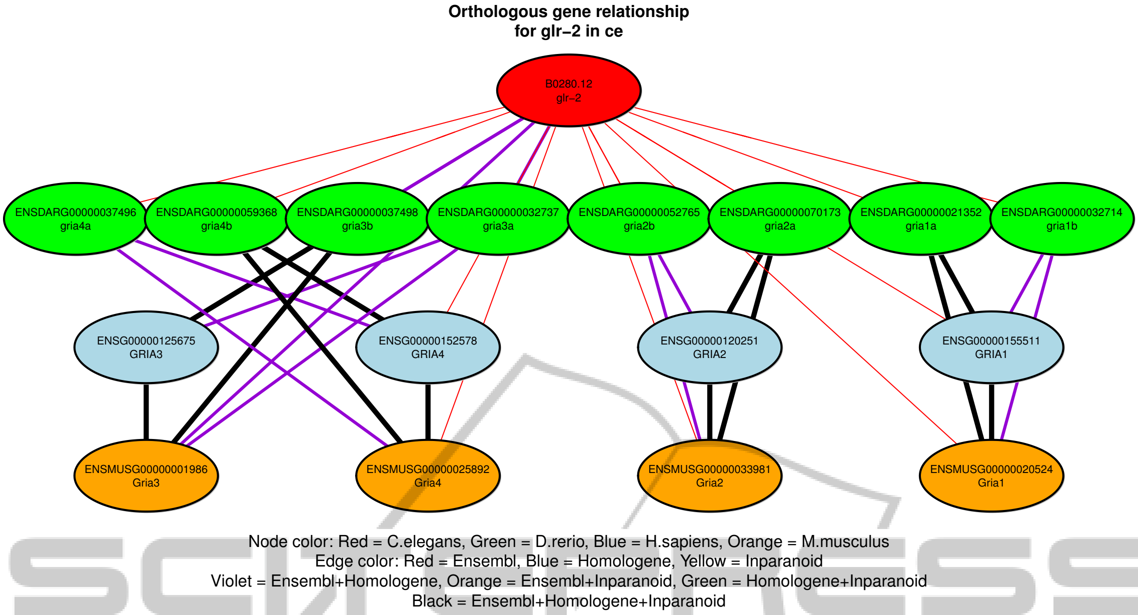


Figure 3: Example R plot for the orthologous relationship of the glr-2 gene in *C.elegans* using the command: `get.orthologe.table("B0280.12","ce",plot=TRUE,self=FALSE)` or `get.orthologe.table(get.gene.info("glr-2","external_gene_id","ensembl_gene_id","ce"), + "ce",plot=TRUE,self=FALSE)`.

in Jena, Germany[1]. In this project, normal and perturbed ageing is investigated by measuring the complete transcriptomes of five organisms (roundworm, zebrafish, killifish, mouse and human) at different age levels using next-generation-sequencing technologies. The goal of this project is to study the complex interplay of maintenance and repair networks in the process of ageing in these organisms. The software introduced in this paper has essentially contributed to the evaluation of the transcriptome data, for instance by clustering temporal expression patterns across species, by enabling a gene set enrichment analysis (GSEA) with four organisms included, and by facilitating the construction of decision trees for gene expression levels by inclusion of multiple species.

In addition, the methods presented here were used in a cross-species comparison (between *M. musculus* and *C. elegans*) of genes that were differentially expressed as a consequence of artificially induced impaired insulin/IGF1 signalling, which extends lifespan in both species. Thereby, six commonly downregulated and thirty commonly upregulated genes could be identified (Zarse et al., 2012).

Another successful application of the orthology package was presented at the RoSyBa[2] in 2011. Here, genes of three species including different tissues, which were differentially expressed during the process of aging were compared using the methods described in this paper, yielding 49 orthologous upregulated (amongst others mod-5, shown in figure 2) and 66 downregulated genes (Fuellen et al., 2012).

3 DISCUSSION

The package presented here is capable of supporting genome-based multi-species approaches where the knowledge of orthology relationships between the genes of two or more organisms is required. Examples for areas of application are systems biology, cancer- or age research. The package subsumes data from three prominent open-access orthology databases, and allows the user to retrieve the information contained in these databases in a consistent manner. By collecting information from several databases, the package gives a more complete picture of orthology compared to each individual database. It allows for selection of robust predictions of orthology if the user confines the search to orthologies predicted by more than one database. The package is free of idle time caused by network traffic because the data related to the investigated organisms is downloaded and processed locally before the package is used for the first time, and is therewith faster than web-based tools. Problems arising from the existence of synonyms for genes or from the occurrence of duplicate

[1] http://www.jenage.de

[2] http://goethe.informatik.uni-rostock.de/ibima/rosyba2011/

gene names and non-unique gene identifiers are overcome by using unique (Ensembl-) identifiers internally. The latter liberates the user from such issues, and in many cases is a prerequisite for a successful orthology search. Orthology relationships between two or more species can be drawn schematically as shown in figure 2, which allows the user to gain insight even if complicated many-to-many orthology relationships between genes prevail.

By default, the current release of the orthology package comprises data for four organisms: *C. elegans*, *D. rerio*, *M. musculus* and *H. sapiens*. The extension towards a larger set of supported species is simply feasible as long as the referring data is available in the HomoloGene database, the Ensembl Compara database, and the Inparanoid database. Future work could be the inclusion of more orthology databases or species into the package. The software is written using the R programming language and is freely available at the Comprehensive R Archive Network - CRAN[3].

4 METHODS

In order to build the package described here, data from different sources, with different structures, had to be combined. The orthology information stored in the databases involved was downloaded, and the datasets were synchronized by cross-mapping the corresponding identifiers used in the individual databases. A diagram showing the databases used and the mappings carried out is depicted in figure 4.

As internal identifiers for the orthology package, Ensembl gene IDs were chosen since two of the included databases work with Ensembl IDs. Information from Ensembl Compara was accessed via the *biomaRt* R package (Durinck et al., 2005) which facilitates connection to the Biomart portal. The HomoloGene database was downloaded in flat-file format[4] and accessed using the R package `annotationTools` (Kuhn et al., 2008). HomoloGene uses Entrez gene IDs as internal identifiers which can be mapped to Ensembl gene IDs using the *biomaRt* package. Data from the Inparanoid database was retrieved with the corresponding homology information R packages from Bioconductor (*e.g.* `hom.Hs.inp.db` for human or `hom.Mm.inp.db` for mouse) (Carlson and Pages, 2012). Inparanoid is based on Ensembl protein IDs and, once again, these IDs were mapped using the `biomaRt` package. The complete datasets were preprocessed and assembled into a compact R package that is run on the local computer independently from online resources. Because of the compact structure, the commands shown in table 1 can be run in parallel on multiple CPUs, although this is not mandatory. The example commands used in this paper only needed a few seconds on a standard desktop computer.

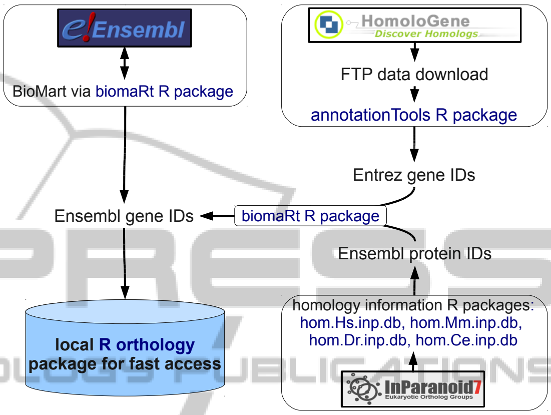

Ensembl
BioMart via biomaRt R package
HomoloGene
Discover Homologs
FTP data download
annotationTools R package
Entrez gene IDs
Ensembl gene IDs
biomaRt R package
Ensembl protein IDs
local R orthology package for fast access
homology information R packages:
hom.Hs.inp.db, hom.Mm.inp.db,
hom.Dr.inp.db, hom.Ce.inp.db
InParanoid7
Eukaryotic Ortholog Groups

Figure 4: Scheme of the accessed databases and of the general data flow.

5 CONCLUSIONS

We present a software package that is able to access and display orthology relationships between multiple species. The software circumvents a number of issues usually connected with orthology searches and provides a convenient and consistent interface to the user. The software has been proven to be useful in several research projects, and is freely available at the Comprehensive R Archive Network (CRAN).

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[3] www.cran.r-project.org

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