Meta-analysis of liver transcriptomic data to identify mammalian functional orthologs

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Abstract

Identifying which orthologs share functions from sequence alone can be challenging, notably in case of paralogous genes families. We hypothesised that co-expression network will help predict functional orthologs amongst complex homologous gene families. To explore the use of transcriptomic data available in public domain to predict functionally equivalent orthologs, we collected genome wide expression data in mouse and rat liver from over 1500 experiments with varied treatments. We used a hyper-graph clustering method to identify clusters of orthologous genes co-expressed in both mouse and rat. We validated these clusters by analysing expression profiles in each species separately, and demonstrating a high overlap. We then focused on genes in 18 homology groups with one-to-many or many-to-many relationships between two species, to discriminate between functionally equivalent and non-equivalent orthologs.

Keywords: gene function, transcriptomics, liver, orthologs, paralogs, co-expression, gene networks

1. Introduction

Annotation of gene function is a crucial step to understand the DNA sequencing data currently generated at an unprecedented rate. The lack of functional annotation forms a major bottleneck in analyses across diverse fields, including *de novo* genome sequencing [1], Genome Wide Association Studies (GWAS) in model and non-model organisms [2], and metagenomics

[3]. An experimental validation of each gene is impractical to this end as it demands high financial and time cost. It is estimated that only one percent of proteins have experimental functional annotations [4]. Bioinformatic approaches therefore provide an attractive alternative [5]. The most widely used and successful gene annotation strategy has been the annotation transfer between homologous genes. Automated annotation pipelines from sequence alone are widely used, including GOtcha [6] and BlastGO [7]. They allow fast annotation of thousands of genes for newly sequenced genomes [8]. This approach can be used within a species, where gene families (paralogs), might share common functions, or across species, where known function(s) of a gene in one species are used to infer functions of the homologous gene(s) in another species.

Despite being widely used, fast computational annotation comes at a cost of misannotation, which is present at high levels (over 10 percent) and is believed to be increasing [9] due to misannotation transfer. The most common misannotation is over-annotation, where a gene is assigned a specific but incorrect function [10]. This is partly because one of the major challenges in functional annotation transfer across species is that the orthology relationships are not always one-to-one. Specifically, a single gene in one species can be homologous to multiple paralogs in another (one-to-many homologies), after gene duplication or gene loss event(s). After a gene duplication, the two paralogs can have redundant functions, and thus would share similar functional annotations, or one copy might diverge (lose functionality, or gain new functionalities, or change cellular localisation or tissue specificity), and thus paralogs should have different functional annotations despite their homology. Similarly, multigene families (with many-to-many homologies) are highly prone to over-annotation errors.

Protein structure information can act as source for functional distinction within multigene family proteins [4]. Protein-protein interaction networks have also been successfully used to identify functional orthologs [11]; two orthologs interacting with the same proteins in each species are likely to share similar functions. Similar strategy has been applied to biochemical pathway information [12]. We here explore the use of co-expression gene networks in this context, as they offer two main advantages over protein-protein interactions and biochemical pathways. First, they can be inferred from transcriptomic datasets, which are more abundant than protein-protein interaction datasets. Second, they allow functional annotation of the various classes of RNA genes. We have previously shown that multi-species

information improves gene network reconstruction [13].

In order to explore the potential of co-expressed gene networks to identify functional equivalents in complex homologous families, we collected transcriptomic data from mouse and rat liver samples. To minimise technical variation, we collected datasets generated using a single microarray platform in each species, resulting into 920 experiments in mouse and 620 experiments in rat. We firstly identified clusters of co-expressed genes using hierarchical clustering and found biologically relevant clusters. We applied a hyper-graph clustering method, SCHype [14] to simultaneously cluster co-expressed orthologous genes between species. We then focussed on 18 complex (one-to-many or many-to-many) homologous groups, where at least one member in mouse and in rat where present in similar co-regulated gene clusters. This allowed the identification of functional homologs in complex homologous families. Our results show the potential of this method to predict functional orthologs and limit over-zealous annotation transfers.

2. Methods

2.1. Data collection and normalisation

Microarray data for liver samples in mouse and rat were collected from GEO, where data for mouse was generated using Affymetrix Mouse Genome 430 2.0 Array, and data for rat was generated using Affymetrix Rat Genome 230 2.0 Array as they were the platforms with a large number of experiments available for each species. Experiments came from 62 (mouse) and 28 (rat) independent studies or GEO series. The GEO accession numbers for individual studies are provided in supplementary table 1. Processed data was not directly comparable between studies, as different studies used different normalisation methods, leading to different distribution of values (supplementary figure 1, A and B). As some datasets had a trimmed lower quartile for reduction in noise by limiting the variability of lowly expressed genes, we applied lower quartile trimming on all datasets (supplementary figure 1, C and D). Specifically, we set the expression value of all probes belonging to the lower quartile to the value of the 25 percentile. We then applied quantile normalisation resulting into a uniform distribution of values for each experiment. To facilitate the comparison between mouse and rat data, we used mouse data as a target for quantile normalisation in rat, using preprocessCore functions `normalize.quantiles.determine.target` and `normalize.quantiles.use.target` [15]. Mouse data was selected as

the target because it contained more experiments than the rat dataset. Thus, after our normalisation steps, the distribution of values was identical for each experiment in both species.

2.2. Data clustering

We selected genes with variable expression across experiments by selecting probes with a standard deviation greater than one across experiments. As shown in figure 1, such probes included genes of low as well as high expression levels, and largely excluded probes showing very low expression in all experiments. Microarray data being already log-transformed, log fold change over the average values were obtained by subtracting the mean expression of each probes.

Hierarchical clustering was done on the log fold change matrices using R functions `dist` ad `hclust` with default parameters (euclidean distance, complete linkage). Dendrogram branches were reordered using the function `order.optimal` from the cba package [16]. Both rows (probes) and columns (experiments) were clustered using this approach.

Gene homology information was retrieved from the Homologen database [17], and probe orthology information was obtained using the R package annotationTools [18]. Due to one-to-many homologs, rat probes and mouse probes intersections resulted into slightly different numbers for each species. Average of the two numbers was used to obtain Jaccard indexes. Jaccard index significance was obtained using the hypergeometric test, and p-values were corrected for multiple testing using Bonferroni correction. Gene ontology enrichment analyses were done using pantherdb [19], using either genes analysed by the microarray or our list of variable genes as a background.

SCHype takes as input a list of conserved interactions which was generated as follows. First Spearman correlation coefficient between each pair of probes was obtained independently for both Mouse and Rat expression data. Pairs of probes with a correlation coefficient greater or equal to 0.5 were selected. Then if orthologs of two connected probes were connected in the other species, they were kept as an SCHype input. SCHype was run with a `minClustsize` of 10, otherwise using default parameters. SCHype identified 132 clusters of homologous genes co-expressed both in mouse and in rat, which included 825 nodes in mouse and 778 nodes in rat. SCHype allows probes to be included in multiple clusters. The different number of probes in mouse and rat is due to the presence of one-to-many and many-to-many

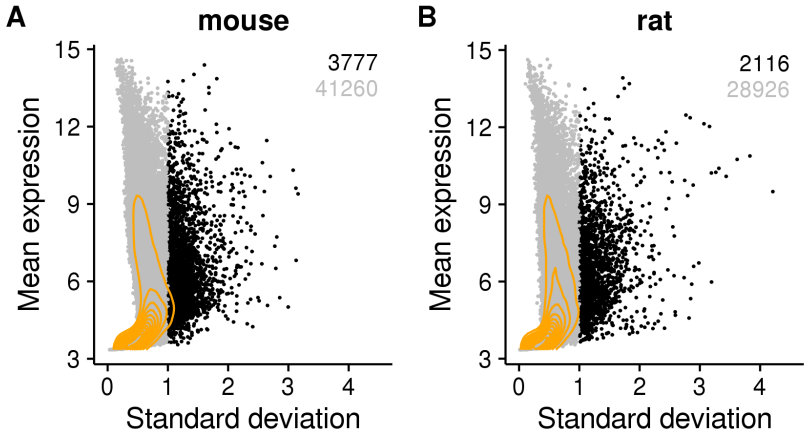

Figure 1: Identification of variable probes in mouse (A) and rat (B) datasets. Each dot represent a single probe. X axis: standard deviation across experiments. Y-axis: mean expression value across experiments (arbitrary units from micro-array processing). In black are the probes with a standard deviation ≥ 1, in grey the probes with a standard deviation < 1. Orange lines: 2D kernel density.

orthologs, as well as the presence of gene measured by multiple probes on the array.

2.3. Scripts and data availability

R scripts used for this analysis are available in a Github repository https://github.com/gdevailly/liver_mouse_rat. Normalised expression matrices, fold change matrices, as well as probe clusters (hierachical clustering and SCHype clustering) are available through a Zenodo collection https://zenodo.org/record/439483.

3. Results

3.1. Identification of variable genes across datasets

We downloaded 920 and 620 experiments for gene expression data in rat and mouse liver from the GEO database. We firstly normalised the data using lower quartile trimming (supplementary figure 1, C and D) and quantile normalization (supplementary figure 1, E and F) independently for each species. We then selected the probes with dynamic expression across samples (standard deviation ≥ 1). This resulted into 3777 probes in mouse

Species	Category	Term	Gene	FE	P-value
Mouse	Reactome	Synthesis of (16-20)-hydroxyeicosatetraenoic acids (HETE)	11	4.78	4.29E-02
		Activation of gene expression by SREBF (SREBP)	15	4.34	5.18E-03
		Regulation of cholesterol biosynthesis by SREBP (SREBF)	17	3.94	4.36E-03
		Cytochrome P450 - arranged by substrate type	27	2.72	7.78E-03
		Phase 1 - Functionalization of compounds	37	2.55	7.58E-04
	GO slim BP	fatty acid metabolic process	52	2.26	2.95E-05
		steroid metabolic process	50	2.18	1.31E-04
Rat	Reactome	Synthesis of bile acids and bile salts via 24-hydroxycholesterol	7	8.63	2.95E-02
		Endosomal/Vacuolar pathway	10	7.93	1.15E-03
		Striated Muscle Contraction	11	6.78	1.48E-03
		ER-Phagosome pathway	10	6.53	6.29E-03
		Activation of gene expression by SREBF (SREBP)	10	6.53	6.29E-03
		Antigen Presentation: Folding, assembly and peptide loading of class I MHC	13	6.27	3.84E-04
		Regulation of cholesterol biosynthesis by SREBP (SREBF)	10	5.55	2.51E-02
		Biological oxidations	25	2.95	3.58E-03
		Metabolism of lipids and lipoproteins	68	2.13	1.15E-05
	GO slim BP	response to biotic stimulus	12	4.16	1.12E-02
		fatty acid metabolic process	22	2.52	2.52E-02

Table 1: Variable genes are enriched for categories and pathways relative to liver functions. FE: Fold enrichment between actual over expected number of genes. GO: gene ontology. BP: biological process. Only category with a fold change higher than 2 are shown. All P-values were corrected for multiple testing with the Bonferroni method.

(8.4%) and 2116 probes in rat (6.8%), with a wide range of expression values (figure 1). 735 mouse variable probes out of 3777 had a homologue in rat variable probes, and 624 rat variable probes out of 2116 had a homologue in mouse variable probes. Variable genes were enriched for pathways and functions relative to liver biology (table 1), including metabolism of lipid an protein (rat, adjusted P value $\leq 10^{-4}$), regulation of cholesterol biosynthesis by SREBP (mouse and rat, respectively adjusted P value ≤ 0.01 and ≤ 0.03), synthesis of bile acid and salt via 24-hydroxycholesterol (rat, adjusted P value ≤ 0.03), and fatty acid metabolic process (mouse and rat, respectively adjusted P value $\leq 10^{-4}$ and ≤ 0.03). As the biological processes enriched in variable genes reflected functions associated with liver, we concluded that the expression variability across samples was reflecting biological variability, and not only technical variations, and therefore was of significance for further investigation.

3.2. Independent hierarchical clustering of mouse and rat data

Hierarchical clustering was applied to the mouse and rat expression matrices independently (figure 2, A and B). We defined 7 major clusters of variable probes, while the experiments were grouped in 4 clusters. The two major clusters of experiments in mouse showed broadly opposite expression patterns (figure 2A). Two major experimental groups were also noted in rat, albeit to a lesser extent compared to mouse (figure 2B). Experiments were annotated according to their series of origin (figure 2A and B, bottom of the heatmap), revealing that most experiments from the same series grouped together (including cases and controls). Notably, no series of experiments were split in the two main experiment clusters.

We characterised the main experiment clusters by looking at the most different non-trivial terms in the element-term matrix build from the metadata retrieved from GEO (characteristic field, figure 2, C and D). No clear difference between experiment clusters was observed in mouse. Experiment cluster 3 in rat seems to be composed mostly of F344 strains of rat and/or of rat treated with the microcystinlr toxin. To note, this cluster is dominated by experiments from a single GSE (figure 2B). Since experiment clustering matched series of origin of the data, this hinders the discrimination of batch effects from biological differences.

Given that mouse and rat probes formed two major clusters anti-correlated with each other despite diverse experimental set ups in each species, we investigated whether the mouse and rat probe clusters were composed of probes measuring similar genes (figure 2E). We calculated the overlap between genes in each cluster in mouse with genes in each cluster in rat. Cluster 2 in mouse (golden color, figure2A) and cluster 2 in rat (golden color, figure 2B) showed a very high overlap with the highest Jaccard index across all clusters. Neither mouse cluster 2 nor rat cluster 2 were enriched for any gene ontology term or reactome pathways, when using the set of variable probes as background. Most clusters did not show a very high genes overlap across species, though the functional enrichment analysis were suggestive that observed gene variations reflected differences in the liver physiology. Specifically, cluster 1 in mouse (claret red color, figure 2A) was enriched for generation of precursor metabolites and energy (adjusted P value $\leq 10^{-6}$), steroid metabolic process (adjusted P value ≤ 0.001), fatty acid metabolic process (adjusted P value ≤ 0.001), and Cytochrome P450 - arranged by substrate type (adjusted P value $\leq 10^{-6}$). Cluster 3 in mouse (green color) was enriched for arachidonic acid metabolic process (adjusted P value ≤ 0.01), icosanoid metabolic

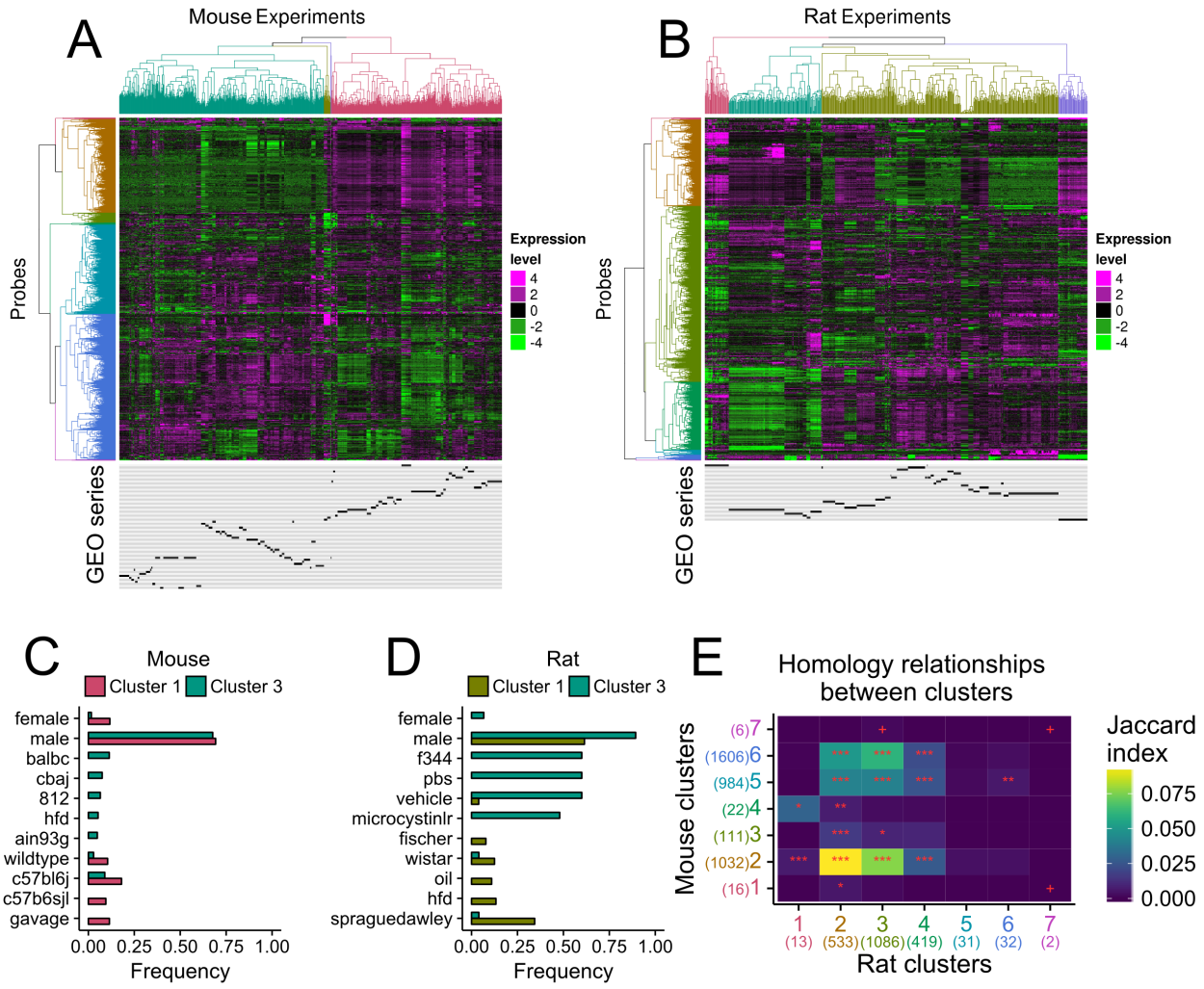

Figure 2: Hierarchical clustering of variable probes in mouse (A) and in rat (B). Four clusters were defined for experiments and seven for probes. Bellow the heatmaps, localisation of experiments from each series were shown in black, one line per series. FC: fold change. C and D. Metadata term frequencies of the two biggest experiment clusters were compared for mouse (C) and rat (D). Colour-code matches the experiments trees in panels A and B. E. Homology relationship between probe clusters between rat (x-axis) and mouse (y-axis). Cell colour: jaccard index. Cell label: Bonferonni adjusted p-values: *** ≤ 0.0001, ** ≤ 0.001, * ≤ 0.01, + ≤ 0.05.

process (adjusted P value ≤ 0.05), fatty acid derivative metabolic process (adjusted P value ≤ 0.05), and Cytochrome P450 - arranged by substrate type (adjusted P value ≤ 0.05). Cluster 6 in rat (blue) was enriched for proteolysis (FE 10, adjusted P value ≤ 0.01). More term relative to the liver matabolism were enriched when the same analysis was used using all genes as a background (supplementary table 2).

3.3. Co-clustering of Mouse and Rat expression data

To identify clusters of homologous probes between mouse and rat, we used the hyper-graph clustering tool SCHype [14]. SCHype uses a recursive spectral clustering algorithm to identify sets of nodes in each species with a greater than expected number of conserved interactions between them (figure 3A). Input data for SCHype was built using three graphs: a mouse probe graph built from pairs of probes with a Spearman correlation coefficient ≥ 0.5 (supplementary figure 2A), a rat probe graph with pairs of probes with a Spearman correlation coefficient ≥ 0.5 (supplementary figure 2B), and a probe to probe homology graph between rat and mouse built using the Homologene database [17] and the annotationTools package [18]. SCHype identified 132 clusters of homologous genes co-expressed both in mouse and in rat, which included 825 nodes in mouse and 778 nodes in rat (figure 3B). SCHype allows probes to be included in multiple clusters resulting into 474 unique probes in mouse and 425 unique probes in rat. It identified four clusters with over 30 homologous genes in each species, eighteen clusters with over 10 probes in each species, thirty-five clusters with only 2 co-expressed probes in each species (figure 3B). We further focussed on the first four (c1-c4) SCHype clusters (figure 3C). We firstly compared SCHype clusters with results obtained by clustering data from each species independently. SCHype cluster c3 highly overlapped with the previous cluster 2 in mouse (golden color, figure 2A) and the cluster 2 in rat (golden color, figure 2B). These two clusters were shown to share a high number of homologous probes (figure 2E). Gene ontology analysis of the four biggest SCHype clusters, both over the set of variable probes or over the full set of probes, did not lead to any significant results, most likely due to small number of genes in each cluster. Importantly, the series experiments in each series no longer clustered together after restricting the data to each of the four biggest SCHype clusters (figure 3C). Individual experiments from each series nevertheless belonged to the same large experiment cluster (figure 3C) highlighting the need for building an expression compendium to obtain these results.

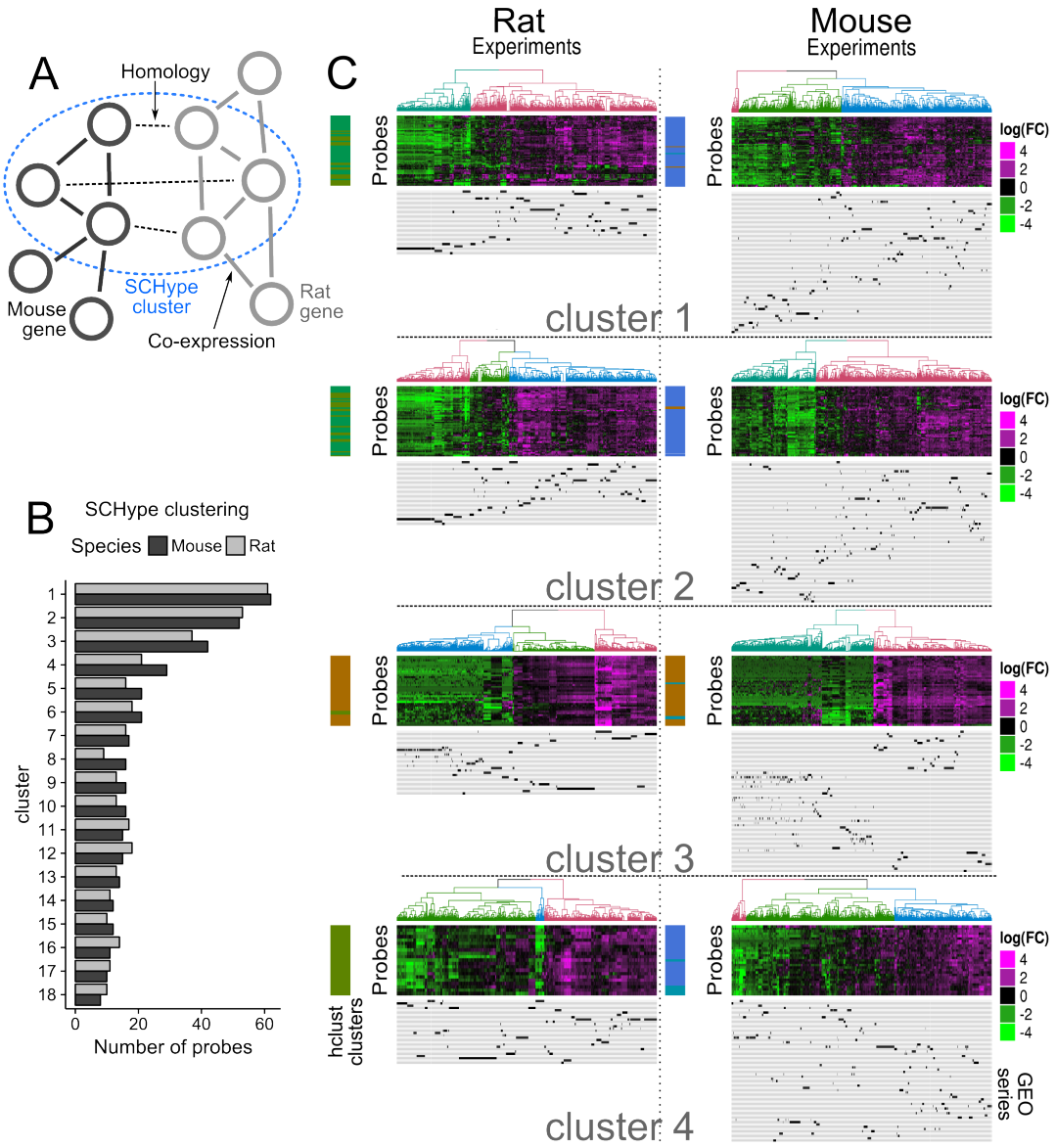

Figure 3: Co-clustering of rat (middle) and mouse (right) data using SCHype. A. SCHype is a clustering tool for hyppergraphs, built here from two co-expression graphs and an homology graph. B. Number of mouse (dark grey) and rat (light grey) probes for the SCHype clusters with more than 10 probes for each species. C. The biggest four SCHype clusters are shown. Genes in mouse and rat in each cluster are homologous to each other. The results of hierarchical clustering for each species is shown as a color bar on the left. Colour-code matches the experiments trees in figure 1. Under the heatmap, clustering localisation of experiments from each series are shown in black, one line per series.

Homology group	Species	Gene name	SCHype cluster	
137229			cluster 69	
	mouse	*Anp32a*	✓	
	rat	*Anp32a*	✓	
	rat	*LOC100909983*		
68982			cluster 7	cluster 30
	mouse	*Ccnb1*	✓	✓
	mouse	*Gm5593*		
	rat	*Ccnb1*	✓	✓
10699			cluster 2	cluster 118
	mouse	*Cd248*	✓	✓
	rat	*Cd248*	✓	✓
	rat	*LOC100911932*		
	rat	*LOC100911882*		
3938			cluster 1	
	mouse	*Ppp1r3c*	✓	
	rat	*Ppp1r3c*	✓	
	rat	*LOC100910671*		
14108			cluster 2	
	mouse	*Rasl10b*	✓	
	rat	*Rasl10b*	✓	
	rat	*LOC100912246*		

Table 2: SCHype clustering of homologous groups: predicted functionality is following gene names. Homology group is from the Homologene database. Tick mark indicates the inclusion of the gene in the corresponding SCHype cluster.

3.4. Co-clustering across species identifies functionally equivalent orthologs

SCHype clustering successfully identified clusters of homologous genes co-expressed in both mouse and rat datasets. This information adds an independent evidence in support of a functional annotation transfer for pairs of orthologous genes across species found in the same SCHype cluster(s), as functionally equivalent orthologs would be co-expressed with the same set of genes in both species, and therefore would be included in the same SCHype cluster(s). We investigated if SCHype clusters could help identify functionally equivalent orthologs amongst complex homology groups. Eighteen homology groups of three members or more had at least one member of each species in the same SCHype cluster(s). For example, for homology group 137299 (table 2), *Anp32a* in mouse and *Anp32a* in rat were in the same SCHype cluster 69, while *LOC100909983*, another homologue of rat *Anp32a*, was not. This suggests that indeed *Anp32a* in rat is the functional equivalent of *Anp32a* in mouse, but *LOC100909983* is not. In this case, our method found back a functional equivalent already known [20]. Similar observations were made for homology groups 68982 (*Ccnb1*), 10699 (*Cdc248*), 3938 (*Ppp1r3c*), and 14108 (*Rasl10b*) (table 2). In five cases, all members of

Homology group	Species	Gene name	SCHype cluster		
128630			cluster 9	cluster 12	cluster 45
	mouse	*Ceacam1*	✓		
	mouse	*Ceacam2*	✓	✓	✓
	rat	*Ceacam1*	✓	✓	✓
11456			cluster 5		
	mouse	*Elovl6*	✓		
	rat	*Elovl6*	✓		
	rat	*LOC102549542*	✓		
20277			cluster 35		
	mouse	*Rrm2*	✓		
	rat	*Rrm2*	✓		
	rat	*LOC100359539*	✓		
55991			cluster 1	cluster 119	
	mouse	*Tmed2*	✓	✓	
	mouse	*Gm21540*	✓	✓	
	rat	*Tmed2*	✓	✓	
11890			cluster 10	cluster 43	cluster 81
	mouse	*Tnks2*	✓	✓	✓
	rat	*LOC100910717*	✓	✓	✓
	rat	*Tnks2*	✓	✓	✓

Table 3: SCHype clustering of homologous groups: all member of the homology groups share predicted functionalities. Homology group is from the Homologene database. Tick mark indicates the inclusion of the gene in the corresponding SCHype cluster.

the homology groups were included in the same SCHype clusters (table 3), suggesting that all orthologs are likely to share the same function(s). Finally, eight homology groups showed more complex situations, where neither only one nor all the homologs where present in the same groups (table 4 and supplementary table 3). For example, in homology group 117945, *Cyp2c7* in rat had three homolous genes in mouse but only *Cyp2c38* in mouse belonged to the same SCHype cluster (table 4) predicting that mouse *Cyp2c38* (and not mouse *Cyp2c29* or mouse *Cyp2c39*) is a functional ortholog of rat *Cyp2c7*.

4. Discussion

Here we have shown that transcriptomic data can be used to help predict functionally orthologous genes, using co-expression networks built from mouse and rat liver samples. We identified 18 complex homologous groups (i.e. with paralogs in at least one of the species), including 54 genes in mouse and 46 genes in rat, with at least one gene in mouse and one gene in rat in the same SCHype cluster(s). Lowering the correlation threshold and the standard deviation threshold will likely increase the number of homologous groups, potentially with a higher false positive rate. In this study, we focussed on a single tissue as a proof of concept. Addition of data from various

Homology group	Species	Gene name	SCHype cluster
117948			cluster 102
	mouse	*Cyp2c38*	✓
	mouse	*Cyp2c29*	
	mouse	*Cyp2c39*	
	rat	*Cyp2c7*	✓
104115			cluster 33
	mouse	*Hsd3b5*	✓
	mouse	*Gm10681*	
	mouse	*Hsd3b4*	
	mouse	*Gm4450*	
	rat	*Hsd3b5*	✓
	rat	*LOC100911116*	✓
137425			cluster 2
	mouse	*Lce3c*	✓
	rat	*LOC100361951*	✓
	rat	*LOC100911982*	✓
	rat	*Lce3d*	
129514			cluster 17
	mouse	*Rdh9*	✓
	mouse	*Rdh1*	
	mouse	*Rdh16*	
	mouse	*Rdh19*	
	mouse	*BC089597*	
	rat	*Rdh16*	✓
	rat	*LOC100365958*	✓

Table 4: SCHype clustering of homologous groups: non-trivial functional relations. Homology group is from the Homologene database. Tick mark indicates the inclusion of the gene in the corresponding SCHype cluster. Four additional, more complex, homology groups are shown in supplementary table 3.

other tissues might lead to co-expressed gene networks of many more genes, but they may lack the fine resolution that is needed to improve functional annotation inference due to lack of a tissue specific context. We used microarray data in this study as it is by far the most abundant dataset. However, consortia like GTEx [21] have generated large amount of RNA sequencing data, and we envisage application of the method described here to RNA sequencing data in the future. The greater sensitivity of RNA sequencing over microarray [22] might allow the identification of more co-expressed genes.

Despite rigorous data normalisation, experiments from the same series tended to cluster together, cases and controls included. While this could be a sign of technical biases, gene ontology analysis of the variable genes demonstrated that they are related to liver functions. Thus it appears that the gene expression variability we observed is, at least partially, reflecting biological variations impacting the liver physiology. Importantly, individual experiments from series did not cluster together in SCHype clusters. We applied various approaches but could not identify the biological origin(s) of the observed variations. This is in part due to the lack of standardised experiment metadata fields in GEO (not all datasets even had a strain or a sex information, for example), and the lack of controlled vocabulary used to describe experiments. It is a possibility that better annotation of the metadata would have allowed the identification of critical confounding factors.

SCHype clustering was able to find some known as well as some yet to be experimentally validated orthologous functional relationships. For example, only mouse and rat *Ccnb1* where in the same SCHype cluster, and not *Gm5593*. While mouse *Ccnb1* and rat *Ccnb1* are annotated as protein coding genes, *Gm5593* in mouse is annotated as a processed pseudogene [20].

Finally, we note that co-expression of orthologous genes is not a validation of shared functionality, but it can be used as another source of evidence. While protein-protein interaction networks could be used for the same aim, transcriptomic data are more easily generated and therefore widely available for many species. Thus the method described here shows a promise to enhance functional gene annotation transfer across species. It can provide an experimental support for one-to-one ortholog annotation transfer, and can help identify functionally similar and non similar orthologs in one-to-many and many-to-many orthology groups.

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