ExprX-vignette

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ExprX is an R package to streamline interspecies differential expression analysis. Taking TPM or FPKM/RPKM files for samples from different species as input, it provides functions to handle all the necessary steps, including data loading, ortholog matching, normalization, differential analysis and visualization.

Using RNA-Seq data for human and mouse brain, this vignette demonstrates how to detect differentially expressed genes between species using ExprX. All the involved steps are described below.

Use the **install_git** function from devtools package to install ExprX from GitHub:

```r
library(devtools)
install_git("https://github.com/mingansun/ExprX")
```

To load the ExprX package:

```r
library(ExprX)
#> Welcome to use ExprX!
```

For more details about how to install and use **ExprX**, please refer to the website: https://github.com/mingansun/ExprX

1. Generate ExprX object by integrating interspecies expression data

By parsing the meta table (as a data frame or CSV file) which contains information about expression data files (usually contain TPM, FPKM or RPKM values) for different species, the function **make_ExprX_dataset** can read these data files to create an object which contains the expression levels of the replicates of different species. The created ExprX object can also contain additional data such as orthologue pairs, normalized expression etc, and will be used by most of the subsequent analysis.

To use **make_ExprX_dataset** to read CSV file with meta data for expression data files and compared species (ie. human and mouse) to create an ExprX object:

```r
# meta table file
hs2mm.meta_file <- paste0(path.package("ExprX"), "/extdata/brain_metatable.csv")

# make ExprX object from meta table
hs2mm.data  <- make_ExprX_dataset(
  hs2mm.meta_file,
  data_dir = paste0(path.package("ExprX"), "/extdata")
  )
#> x is detected as a file name. Read to a data frame:
#> Species  FullName    AbbrName    IdColumn    ExprColumn  ExprType    RepIndex    File
#> human    Homo Sapiens    hsapiens    1   6   tpm 1   human_brain_1.genes.results
#> human    Homo Sapiens    hsapiens    1   6   tpm 2   human_brain_2.genes.results
#> human    Homo Sapiens    hsapiens    1   6   tpm 3   human_brain_3.genes.results
#> mouse    mmusculus   mmusculus   1   6   tpm 1   mouse_brain_1.genes.results
#> mouse    mmusculus   mmusculus   1   6   tpm 2   mouse_brain_2.genes.results
#> mouse    mmusculus   mmusculus   1   6   tpm 3   mouse_brain_3.genes.results
#>
#> Read gene IDs for each species ...
#> human: 1042 genes.
#> mouse: 1037 genes.
#>
#> Loading expression data for each species ...
#> human: 1042 genes from 3 files.
#> mouse: 1037 genes from 3 files.
```

2. Determine the 1-to-1 orthologs among compared species

The 1-to-1 orthologs among species are constructed based on the homolog annotations from ENSEMBL database. Thus, only species available in in ENSEMBL database (about 200 as checked on 2020-4-7) can be used for analysis.

To use the **list_species** function to get the information (eg. species name, abbreviation) for all the species supported by ExprX:

```
sp.lst <- list_species()
head(sp.lst)
#>         Dataset       Species                Version
#> 1   acalliptera Eastern happy            fAstCal1.2
#> 2 acarolinensis  Anole lizard             AnoCar2.0
#> 3  acchrysaetos  Golden eagle            bAquChr1.2
#> 4  acitrinellus Midas cichlid              Midas_v5
#> 5  amelanoleuca         Panda               ailMel1
#> 6    amexicanus Mexican tetra Astyanax_mexicanus-2.0
```

Match and save 1-to-1 orthologs among species

The **ortholog_match** function invokes **biomaRt** package to retrieve homolog annotation from ENSEMBL database, then matches 1-to-1 orthologs by reciprocal comparison. This step usually takes a few minutes - depending on the network speed). To speed up, the obtained ortholog data can be stored on hard disk with **saveRDS** for later use.

```
# Match 1:1 orthologs between human and mouse
hs2mm.orth <- ortholog_match("human", "mouse")

# Save the ortholog results on hard disk for later use
saveRDS(hs2mm.orth, "hs2mm.orth.rds")
```

Load previously saved ortholog matching results from hard disk

Below shows how to used **readRDS** to load ortholog matching result that is previously saved on hard disk. Alternatively, the ortholog matching result can be generated with **ortholog_match** as demonstrated above.

The ortholog matching result includes information for each involved species, such as GeneID, GeneName, Chrom, GeneType and so on.

```
# Load ortholog result with readRDS
hs2mm.orth <- readRDS(paste0(path.package("ExprX"), "/data/hs2mm.orth.rds"))

# View the structure of hs2mm.orth
str(hs2mm.orth)
#> List of 2
#>  $ human:'data.frame':   16536 obs. of  4 variables:
#>   ..$ GeneID  : chr [1:16536] "ENSG00000198695" "ENSG00000198712" "ENSG00000198727"
  "ENSG00000198763" ...
#>   ..$ GeneName: chr [1:16536] "MT-ND6" "MT-CO2" "MT-CYB" "MT-ND2" ...
#>   ..$ Chrom   : chr [1:16536] "MT" "MT" "MT" "MT" ...
#>   ..$ GeneType: chr [1:16536] "protein_coding" "protein_coding" "protein_coding" "protein_coding"
  ...
#>  $ mouse:'data.frame':   16536 obs. of  4 variables:
#>   ..$ GeneID  : chr [1:16536] "ENSMUSG00000064368" "ENSMUSG00000064354" "ENSMUSG00000064370"
  "ENSMUSG00000064345" ...
#>   ..$ GeneName: chr [1:16536] "mt-Nd6" "mt-Co2" "mt-Cytb" "mt-Nd2" ...
#>   ..$ Chrom   : chr [1:16536] "MT" "MT" "MT" "MT" ...
#>   ..$ GeneType: chr [1:16536] "protein_coding" "protein_coding" "protein_coding" "protein_coding"
  ...
#>  - attr(*, "Species")= chr [1:2] "human" "mouse"
#>  - attr(*, "SpeciesAbbr")= chr [1:2] "hsapiens" "mmusculus"
#>  - attr(*, "SpeciesFull")= chr [1:2] "Homo Sapiens" "mmusculus"
```

To summarize the ortholog results by genetype (eg. protein_coding, miRNA, lncRNA etc):

```
hs2mm.orth.genetype <- summarize_ortholog_gene(hs2mm.orth, group = "genetype")
head(hs2mm.orth.genetype)
#>                          human mouse
#> IG_C_gene                    3     3
#> IG_V_gene                    2     2
#> lncRNA                       1     0
#> miRNA                      218   218
#> misc_RNA                    72    73
#> polymorphic_pseudogene      21    15
```

To summarize the ortholog matching results by chromosome:

```
hs2mm.orth.chrom <- summarize_ortholog_gene(hs2mm.orth, group = "chrom")
head(hs2mm.orth.chrom)
#>    human mouse
#> 1   1727  1050
#> 10   636   779
#> 11  1048  1342
#> 12   889   565
#> 13   286   546
#> 14   572   588
```

Filter 1-to-1 orthologs to exclude specific groups of genes

In many cases, specific groups of genes (eg. pseudogenes or genes from sex chromosomes) are undesirable for gene expression comparison. The function **ortholog_filter** enables the filtering of ortholog pairs based on gene type, chromosome, or provided gene list, as demonstrated below.

```
# Filter orthologs by excluding genes from chromosomes X, Y and MT, and only keep
# protein_coding genes
hs2mm.orth.flt <- ortholog_filter(
  hs2mm.orth,
  genetype_include = "protein_coding",
  chrom_exclude = c("X", "Y", "MT")
)
#> Check data for human
#> genetype_include matches:    15849
#> chrom_exclude    matches:    595
#>
#> Check data for mouse
#> genetype_include matches:    15855
#> chrom_exclude    matches:    590
#>
#> Original gene number: 16536
#> Filtered gene number: 15275
```

To check the number of 1:1 orthologs before and after filtering:

```
# Before filtering
dim(hs2mm.orth[[1]])
#> [1] 16536     4

# After filtering
dim(hs2mm.orth.flt[[1]])
#> [1] 15275     4
```

3. Integrate the 1-to-1 ortholog matching result to ExprX object

Take the original ExprX object generated with **make_ExprX_dataset** and the ortholog matching data generated using **ortholog_match** as input, the function **ortholog_expression_merge** integrates together the expression data for all 1:1 orthologs among compared species. The integrated data will be appended to the original ExprX object and returned as an updated object. To be noted, for orthologs that don’t have matched expression data, they will be excluded from the returned data.

```
# Merge data
hs2mm.data <- ortholog_expression_merge(
  expr_data = hs2mm.data, orth_data = hs2mm.orth.flt
)
#> Number of ortholog pairs absent from expr_data
#> human:   14233
#> mouse:   14238
#>
```

```
#> Original  ortholog number: 15275
#> Discarded ortholog number: 14375
#> Resulted  ortholog number: 900
```

4. Integrate the normalized expression data for 1-to-1 orthologs to ExprX object

Normalization of the expression data for 1:1 orthologs among species can be performed by using the **ortholog_expression_normalize** function. Different normalization approaches are supported, including TMM, TMMwsp, RLE, upperquartile and quantile. The normalized data matrix will be appended to the original ExprX object and returned as the updated ExprX object.

To normalize the expression data of 1:1 orthologs among samples using the TMM approach:

```
# Load required package
library(edgeR)
#> Loading required package: limma

# Perform data normalization
hs2mm.data <- ortholog_expression_normalize(
  expr_data = hs2mm.data, method = "TMM"
)
```

5. Perform interspecies differential expression analysis

Differential expression analysis of 1:1 orthologs between species can be performed using the **ortholog_expression_compare** function. Expression data of 1:1 orthologs after normalization are used for differential expression analysis. Statistics such as average expression level, log2foldChange and p-values are calculated and returned as a dataframe.

Below shows how to perform interspecies differential analysis for 1:1 orthologs using RankProd approach. To be noted, the demo dataset is only for less than 1000 genes, thus only a couple of genes are called as differentlly expressed. If use full dataset, the number of called differential genes can be as many as several hundreds.

```
# Load required package
library(RankProd)
#> Loading required package: Rmpfr
#> Loading required package: gmp
#>
#> Attaching package: 'gmp'
#> The following objects are masked from 'package:base':
#>
#>     %*%, apply, crossprod, matrix, tcrossprod
#> C code of R package 'Rmpfr': GMP using 64 bits per limb
#>
#> Attaching package: 'Rmpfr'
#> The following object is masked from 'package:gmp':
#>
#>     outer
#> The following objects are masked from 'package:stats':
#>
#>     dbinom, dgamma, dnorm, dpois, pnorm
#> The following objects are masked from 'package:base':
#>
#>     cbind, pmax, pmin, rbind

# Perform differential analysis and then sort by p-value
hs2mm.deg <- ortholog_expression_compare(
  hs2mm.data, method = "RankProd", p_adjust = "fdr"
  )
#> Rank Product analysis for unpaired case
#>
#>
#>  done
hs2mm.deg <- hs2mm.deg[order(hs2mm.deg$P_value),]

# Check what the differential analysis result looks like
head(hs2mm.deg)
#>         GeneID_human       GeneID_mouse GeneName_human GeneName_mouse
#> 233 ENSG00000131095 ENSMUSG00000020932           GFAP           Gfap
#> 256 ENSG00000197971 ENSMUSG00000041607            MBP            Mbp
#> 316 ENSG00000120885 ENSMUSG00000022037            CLU            Clu
#> 858 ENSG00000167996 ENSMUSG00000024661           FTH1           Fth1
#> 396 ENSG00000136161 ENSMUSG00000022106         RCBTB2         Rcbtb2
#> 674 ENSG00000120094 ENSMUSG00000018973          HOXB1          Hoxb1
#>     Expression_human Expression_mouse Expression_average log2foldChange
#> 233       98707.9359         1.947577         49354.9418      15.299531
#> 256      207093.3485        91.182026        103592.2653      11.141358
#> 316      137866.0153       345.075280         69105.5453       8.640056
#> 858       99991.8187      3601.512987         51796.6659       4.794942
#> 396         244.2676     34316.340494         17280.3041      -7.131360
#> 674           0.0000       276.888419           138.4442      -9.115764
#>         P_value
#> 233 0.009575705
#> 256 0.009575705
#> 316 0.016379287
#> 858 0.037521803
#> 396 0.047574220
#> 674 0.047574220
```

To determine differential genes (based on cufoff of p-value and log2foldChange) which can be saved for downstream analysis:

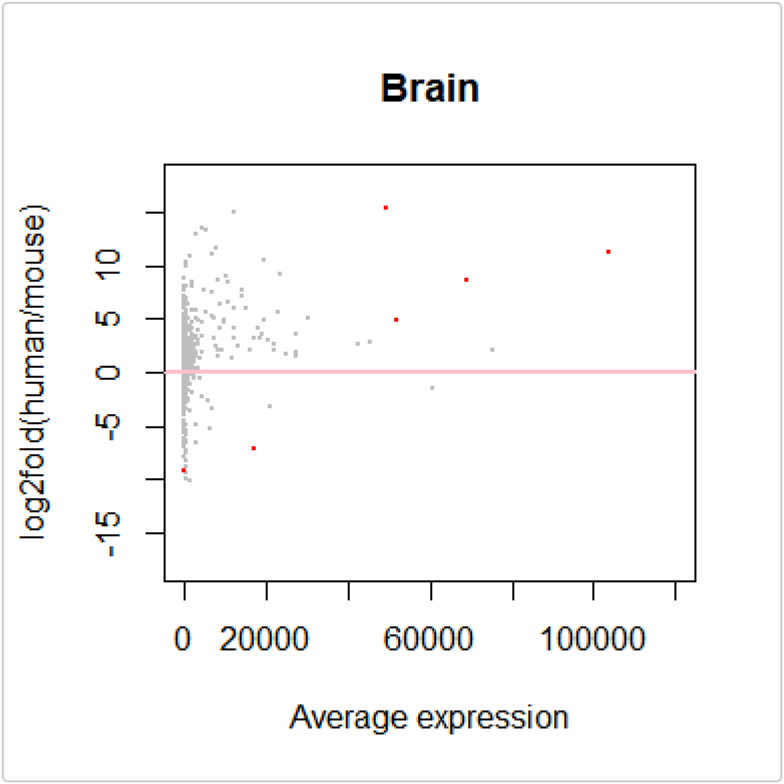
```
# Determine significant differential genes (human>mouse) based on p-values and log2foldChange
hs2mm.hsHigh <- subset(hs2mm.deg, subset = log2foldChange > 1  & P_value < 0.05)
head(hs2mm.hsHigh)
#>         GeneID_human       GeneID_mouse GeneName_human GeneName_mouse
#> 233 ENSG00000131095 ENSMUSG00000020932           GFAP           Gfap
#> 256 ENSG00000197971 ENSMUSG00000041607            MBP            Mbp
#> 316 ENSG00000120885 ENSMUSG00000022037            CLU            Clu
#> 858 ENSG00000167996 ENSMUSG00000024661           FTH1           Fth1
#>     Expression_human Expression_mouse Expression_average log2foldChange
#> 233        98707.94        1.947577          49354.94      15.299531
#> 256       207093.35       91.182026         103592.27      11.141358
#> 316       137866.02      345.075280          69105.55       8.640056
#> 858        99991.82     3601.512987          51796.67       4.794942
#>         P_value
#> 233 0.009575705
#> 256 0.009575705
#> 316 0.016379287
#> 858 0.037521803
```

```
# Determine significant differential genes (human<mouse) based on p-values and log2foldChange
hs2mm.mmHigh <- subset(hs2mm.deg, subset = log2foldChange < -1 & P_value < 0.05)
head(hs2mm.mmHigh)
#>        GeneID_human       GeneID_mouse GeneName_human GeneName_mouse
#> 396 ENSG00000136161 ENSMUSG00000022106         RCBTB2         Rcbtb2
#> 674 ENSG00000120094 ENSMUSG00000018973          HOXB1          Hoxb1
#>     Expression_human Expression_mouse Expression_average log2foldChange
#> 396         244.2676       34316.3405         17280.3041      -7.131360
#> 674           0.0000         276.8884           138.4442      -9.115764
#>        P_value
#> 396 0.04757422
#> 674 0.04757422
```

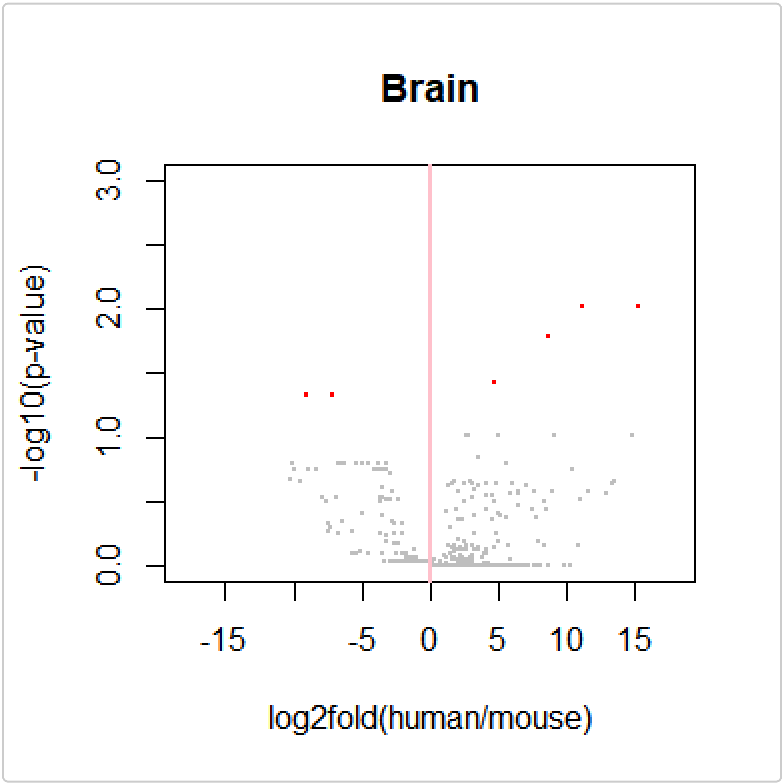
6. Visualize differential expression analysis result

The differential expression can be visualized as MA-plot or Volcano-plot, with differential genes highlighted by color. Below shows how to use the **ortholog_expression_plot** function to generate MA-plot and Volcano-plot, respectively.

```
# Generate MA-plot
ortholog_expression_plot(
  hs2mm.deg, "MA",
  main = "Brain", xlim = c(0,120000), ylim = c(-18,18)
  )
```



```
# Generate Volcano-plot
ortholog_expression_plot(
  hs2mm.deg, "volcano",
  main = "Brain", xlim = c(-18,18), ylim = c(0, 3)
  )
```


Brain
-log10(p-value)
log2fold(human/mouse)