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## Contents

1	Introd	duction	2			
2	Data loading: species trees and metadata					
3	Identification and classification of duplicated genes in Ensembl and Ensembl Genomes					
	3.1	Ensembl Fungi	3			
	3.2	Ensembl Protists	4			
	3.3	Ensembl Plants	4			
	3.4	Ensembl Metazoa	6			
	3.5	Ensembl (Vertebrates)	8			
	Sessi	ion info	10			

## 1 Introduction

Here, we will describe the code to identify and classify duplicated genes in Ensembl and Ensembl Genomes species using the Bioconductor package *doubletrouble*.

```
library(syntenet)
library(doubletrouble)
library(biomaRt)
library(here)
## here() starts at /home/faalm/Dropbox/package_benchmarks/doubletrouble_paper
library(tidyverse)
## -- Attaching core tidyverse packages ----- tidyverse 2.0.0 --
## v dplyr 1.1.4 v readr 2.1.5
## v forcats 1.0.0 v stringr 1.5.1
## v ggplot2 3.4.4 v tibble 3.2.1
## v lubridate 1.9.3
                       v tidyr
                                  1.3.1
             1.0.2
## v purrr
## -- Conflicts ----- tidyverse_conflicts() --
## x dplyr::filter() masks stats::filter()
## x dplyr::lag() masks stats::lag()
## x dplyr::select() masks biomaRt::select()
## i Use the conflicted package (<a href="http://conflicted.r-lib.org/">http://conflicted.r-lib.org/</a>) to force all conflicts to become errors
set.seed(123) # for reproducibility
options(timeout = 1e10) # to allow download of big files
# Load helper functions
source(here("code", "utils.R"))
```

## 2 Data loading: species trees and metadata

Here, we will load the data frames of species metadata and phylo objects with species trees for each Ensembl instance.

```
# Load metadata
load(here("products", "result_files", "metadata_all.rda"))
names(metadata_all)
## [1] "fungi" "plants" "metazoa" "protists" "ensembl"

# Load trees
load(here("products", "result_files", "trees", "fungi_busco_trees.rda"))
load(here("products", "result_files", "trees", "plants_busco_trees.rda"))
load(here("products", "result_files", "trees", "metazoa_busco_trees.rda"))
load(here("products", "result_files", "trees", "vertebrates_busco_trees.rda"))
load(here("products", "result_files", "trees", "protists_busco_trees.rda"))
```

## 3 Identification and classification of duplicated genes in Ensembl and Ensembl Genomes

Now, let's use *doubletrouble* to identify duplicated genes and classify them using the Ensembl and Ensembl Genomes data sets. Here, to avoid code repetition and optimize memory usage, we will use the wrapper function ensembl2duplicates() (in the file utils.R). For each species in the metadata data frame, this function:

- Retrieves whole-genome protein sequences (AAStringSet) and gene annotation (GRanges) from an Ensembl instance;
- 2. Filters the AAStringSet object to include only the longest protein for each gene (i.e., the translated sequence of the primary transcript);
- 3. Processes the sequences and annotation with syntenet::process\_input();
- 4. Identifies the paranome with syntenet::run\_diamond() + identifies orthologs between the query species and an outgroup (optional);
- 5. Classifies paralogs by duplication modes.

## 3.1 Ensembl Fungi

First, let's create a data frame with species and their outgroups. Here, we will use the basid-iomycete *Cryptococcus neoformans* as outgroup for Ascomycota species, and the oomycete *Aphanomyces astaci* as outgroup for Basidiomycota species.

```
col_dir <- here("products", "result_files", "collinearity", "fungi")</pre>
if(!dir.exists(col_dir)) { dir.create(col_dir, recursive = TRUE) }
# Create data frame of query species and outgroup
fungi_outgroups <- metadata_all$fungi |>
    filter(phylum != "Oomycota") |>
    mutate(
        query = species,
        outgroup = case_when(
            phylum == "Ascomycota" ~ "cryptococcus_neoformans",
            TRUE ~ "aphanomyces_astaci"
    ) |>
    select(query, outgroup)
# Identifying and classifying paralogs
fungi_duplicates <- ensembl2duplicates(</pre>
    metadata_all$fungi, ensembl = "fungi",
    outgroups = fungi_outgroups,
    collinearity_dir = col_dir
)
# Classify genes into unique duplication modes
fungi_duplicates_unique <- classify_genes(fungi_duplicates)</pre>
# Save classification results
```

```
## Duplicate pairs
save(
    fungi_duplicates,
    file = here("products", "result_files", "fungi_duplicates.rda"),
    compress = "xz"
)

## Duplicated genes (unique duplication modes)
save(
    fungi_duplicates_unique,
    file = here("products", "result_files", "fungi_duplicates_unique.rda"),
    compress = "xz"
)
```

#### 3.2 Ensembl Protists

Since protists are not a real (i.e., monophyletic) group, defining an outgroup species is very problematic. For this reason, we will classify duplicates using the *standard* classification scheme here.

```
col_dir <- here("products", "result_files", "collinearity", "protists")</pre>
if(!dir.exists(col_dir)) { dir.create(col_dir, recursive = TRUE) }
# Identifying and classifying paralogs
protists_duplicates <- ensembl2duplicates(</pre>
    metadata_all$protists, ensembl = "protists", collinearity_dir = col_dir
# Classify genes into unique duplication modes
protists_duplicates_unique <- classify_genes(protists_duplicates)</pre>
# Save classification results
## Duplicate pairs
save(
    protists_duplicates,
    file = here("products", "result_files", "protists_duplicates.rda"),
    compress = "xz"
)
## Duplicated genes (unique duplication modes)
    protists_duplicates_unique,
    file = here("products", "result_files", "protists_duplicates_unique.rda"),
    compress = "xz"
)
```

#### 3.3 Ensembl Plants

Here, we will use different outgroups for different branches of the tree. The clades and outgroups are:

- 1. Angiosperms: Amborella trichopoda as outgroup.
- 2. Amborella trichopoda and Nymphaea colorata: Chara braunii as outgroup.
- 3. Selaginella moellendorffii, Chara braunii, Marchantia polymorpha, Physcomitrium patens: Chlamydomonas reinhardtii as outgroup.
- 4. Chlamydomonas reinhardtii and Ostreococcus lucimarinus: Galdieria sulphuraria as outgroup
- 5. Rhodophyta algae: no outgroup.

```
# Create data frame of guery species and outgroup
angiosperms <- metadata_all$plants |>
    filter(
        phylum == "Streptophyta",
        !order %in% c(
            "Charales", "Selaginellales", "Funariales",
            "Marchantiales", "Nymphaeales"
        )
    ) |>
    pull(species)
ana <- c("amborella_trichopoda", "nymphaea_colorata")</pre>
bryophytes <- c(
    "selaginella_moellendorffii", "chara_braunii",
    "marchantia_polymorpha", "physcomitrium_patens"
chlorophyta <- c("chlamydomonas_reinhardtii", "ostreococcus_lucimarinus")</pre>
plants_outgroups <- metadata_all$plants |>
    filter(phylum != "Rhodophyta") |>
    mutate(
        query = species,
        outgroup = case_when(
            species %in% angiosperms ~ "amborella_trichopoda",
            species %in% ana ~ "chara_braunii",
            species %in% bryophytes ~ "chlamydomonas_reinhardtii",
            species %in% chlorophyta ~ "galdieria_sulphuraria"
    ) |>
    select(query, outgroup)
```

#### Identifying and classifying duplicates:

```
col_dir <- here("products", "result_files", "collinearity", "plants")
if(!dir.exists(col_dir)) { dir.create(col_dir, recursive = TRUE) }

# Identifying and classifying paralogs
plants_duplicates <- ensembl2duplicates(
    metadata_all$plants, ensembl = "plants",
    outgroups = plants_outgroups,
    collinearity_dir = col_dir,
    threads = 4</pre>
```

```
# Classify genes into unique duplication modes
plants_duplicates_unique <- classify_genes(plants_duplicates)

# Save classification results
## Duplicate pairs
save(
    plants_duplicates,
    file = here("products", "result_files", "plants_duplicates.rda"),
    compress = "xz"
)

## Duplicated genes (unique duplication modes)
save(
    plants_duplicates_unique,
    file = here("products", "result_files", "plants_duplicates_unique.rda"),
    compress = "xz"
)</pre>
```

#### 3.4 Ensembl Metazoa

Here, we will use different outgroups for different branches of the tree. The clades and outgroups are:

- 1. Arthropoda: Hypsibius exemplaris (Tardigrada) as outgroup.
- 2. Tardigrada, Acanthocephala, and Rotifera: Brugia malayi (Nematoda) as outgroup.
- 3. Nematoda: Priapulus caudatus (Priapulida) as outgroup
- 4. Priapulida, Echinodermata, Chordata, and Hemichordata: *Hofstenia miamia* (Xenacoelomorpha) as outgroup.
- 5. Xenacoelomorpha: Actinia tenebrosa (Cnidaria) as outgroup.
- 6. Cnidaria and Placozoa: Amphimedon queenslandica (Porifera) as outgroup.
- 7. Porifera: Mnemiopsis leidyi (Ctenophora) as outgroup.
- 8. Brachiopoda: Haliotis rufescens (Mollusca) as outgroup.
- 9. Mollusca, Annelida, and Platyhelminthes: Adineta vaga (Rotifera) as outgroup.

```
# Create data frame of query species and outgroup
by_phylum <- function(df, taxon) {
    return(
          df |>
               dplyr::filter(phylum == taxon) |>
                dplyr::pull(species)
    )
}
arthropoda <- by_phylum(metadata_all$metazoa, "Arthropoda")
tardigrada <- by_phylum(metadata_all$metazoa, "Tardigrada")</pre>
```

```
nematoda <- by_phylum(metadata_all$metazoa, "Nematoda")</pre>
priapulida <- by_phylum(metadata_all$metazoa, "Priapulida")</pre>
xenacoelomorpha <- by_phylum(metadata_all$metazoa, "Xenacoelomorpha")</pre>
cnidaria <- by_phylum(metadata_all$metazoa, "Cnidaria")</pre>
placozoa <- by_phylum(metadata_all$metazoa, "Placozoa")</pre>
porifera <- by_phylum(metadata_all$metazoa, "Porifera")</pre>
brachiopoda <- by_phylum(metadata_all$metazoa, "Brachiopoda")</pre>
mollusca <- by_phylum(metadata_all$metazoa, "Mollusca")</pre>
echinodermata <- by_phylum(metadata_all$metazoa, "Echinodermata")</pre>
annelida <- by_phylum(metadata_all$metazoa, "Annelida")</pre>
platyhelminthes <- by_phylum(metadata_all$metazoa, "Platyhelminthes")</pre>
acanthocephala <- by_phylum(metadata_all$metazoa, "Acanthocephala")</pre>
chordata <- by_phylum(metadata_all$metazoa, "Chordata")</pre>
hemichordata <- by_phylum(metadata_all$metazoa, "Hemichordata")</pre>
rotifera <- by_phylum(metadata_all$metazoa, "Rotifera")</pre>
metazoa_outgroups <- metadata_all$metazoa |>
    filter(phylum != "Ctenophora") |>
    mutate(
        query = species,
        outgroup = case_when(
            species %in% arthropoda ~ "hypsibius_exemplaris_gca002082055v1",
            species %in% c(tardigrada, acanthocephala, rotifera) ~ "brugia_malayi",
            species %in% nematoda ~ "priapulus_caudatus_gca000485595v2",
            species %in% c(priapulida, echinodermata, chordata, hemichordata) ~
                 "hofstenia_miamia",
            species %in% xenacoelomorpha ~ "actinia_tenebrosa_gca009602425v1",
            species %in% c(cnidaria, placozoa) ~
                 "amphimedon_queenslandica_gca000090795v2rs",
            species %in% porifera ~ "mnemiopsis_leidyi",
            species %in% brachiopoda ~ "haliotis_rufescens_gca023055435v1rs",
            species %in% c(mollusca, annelida, platyhelminthes) ~ "adineta_vaga"
        )
    ) |>
    select(query, outgroup)
```

#### Identifying and classifying duplicates:

```
col_dir <- here("products", "result_files", "collinearity", "metazoa")
if(!dir.exists(col_dir)) { dir.create(col_dir, recursive = TRUE) }

# Identifying and classifying paralogs
metazoa_duplicates <- ensembl2duplicates(
    metadata = metadata_all$metazoa,
    ensembl = "metazoa",
    outgroups = metazoa_outgroups,
    collinearity_dir = col_dir,
    threads = 4
)</pre>
```

```
# Classify genes into unique duplication modes
metazoa_duplicates_unique <- classify_genes(metazoa_duplicates)

# Save classification results
## Duplicate pairs
save(
    metazoa_duplicates,
    file = here("products", "result_files", "metazoa_duplicates.rda"),
    compress = "xz"
)

## Duplicated genes (unique duplication modes)
save(
    metazoa_duplicates_unique,
    file = here("products", "result_files", "metazoa_duplicates_unique.rda"),
    compress = "xz"
)</pre>
```

## 3.5 Ensembl (Vertebrates)

Here, we will use the following outgroups per taxa:

- 1. Amniota: Xenopus tropicalis (Amphibia) as outgroup;
- 2. Amphibia: Latimeria chalumnae (West Indian Ocean coelacanth)
- 3. All bony and cartilaginous fish: Eptatretus burgeri (hagfish, Agnatha)
- 4. Agnatha: Ciona intestinalis (Tunicata)

```
# Create a data frame of species and outgroups
amniota <- metadata_all$ensembl |>
    filter(
        class %in% c("Aves", "Mammalia", "Lepidosauria") |
            order %in% c("Testudines", "Crocodylia")
    ) |>
    pull(species)
amphibia <- metadata_all$ensembl |>
    filter(class == "Amphibia") |>
    pull(species)
fish <- metadata_all$ensembl |>
    filter(
        class %in% c("Actinopteri", "Chondrichthyes", "Cladistia") |
            order == "Coelacanthiformes"
    ) |>
    pull(species)
agnatha <- metadata_all$ensembl |>
    filter(
        class %in% c("Myxini", "Hyperoartia")
    ) |>
    pull(species)
```

```
ensembl_outgroups <- metadata_all$ensembl |>
    filter(!phylum %in% c("Nematoda", "Arthropoda", "Ascomycota")) |>
    mutate(
        query = species,
        outgroup = case_when(
            species %in% amniota ~ "xenopus_tropicalis",
            species %in% amphibia ~ "latimeria_chalumnae",
            species %in% fish ~ "eptatretus_burgeri",
            species %in% agnatha ~ "ciona_intestinalis"
        )
        ) |>
        select(query, outgroup) |>
        filter(!is.na(outgroup))
```

#### Identifying and classifying duplicates:

```
col_dir <- here("products", "result_files", "collinearity", "vertebrates")</pre>
if(!dir.exists(col_dir)) { dir.create(col_dir, recursive = TRUE) }
# Identifying and classifying paralogs
vertebrates_duplicates <- ensembl2duplicates(</pre>
    meta,
    ensembl = "ensembl",
    outgroups = ensembl_outgroups,
    collinearity_dir = col_dir,
    tsv_dir = "~/Documents/vertebrates_duplicates", # delete later
    threads = 4
)
# Classify genes into unique duplication modes
vertebrates_duplicates_unique <- classify_genes(vertebrates_duplicates)</pre>
# Save classification results
## Duplicate pairs
save(
    vertebrates_duplicates,
    file = here("products", "result_files", "vertebrates_duplicates.rda"),
    compress = "xz"
## Duplicated genes (unique duplication modes)
save(
    vertebrates_duplicates_unique,
    file = here("products", "result_files", "vertebrates_duplicates_unique.rda"),
    compress = "xz"
```

## Session info

This document was created under the following conditions:

```
## - Session info ------
## setting value
## version R version 4.3.2 (2023-10-31)
## os Ubuntu 22.04.3 LTS
## system x86_64, linux-gnu
## ui X11
## language (EN)
## collate en_US.UTF-8
## ctype en_US.UTF-8
## tz Europe/Brussels
## date 2024-02-27
## pandoc 3.1.1 @ /usr/lib/rstudio/resources/app/bin/quarto/bin/tools/ (via rmarkdown)
##
## package * version date (UTC) lib source
## abind
              1.4-5 2016-07-21 [1] CRAN (R 4.3.2)
```

##	evaluate	0.23	2023-11-01 [1] CRAN (R 4.3.2)	
##	fansi	1.0.6	2023-12-08 [1] CRAN (R 4.3.2)	
##	fastmap	1.1.1	2023-02-24 [1] CRAN (R 4.3.2)	
##	filelock	1.0.3	2023-12-11 [1] CRAN (R 4.3.2)	
##	forcats *	1.0.0	2023-01-29 [1] CRAN (R 4.3.2)	
##	foreach	1.5.2	2022-02-02 [1] CRAN (R 4.3.2)	
##	generics	0.1.3	2022-07-05 [1] CRAN (R 4.3.2)	
##	GenomeInfoDb	1.38.6	2024-02-08 [1] Bioconductor 3.18 (F	R 4.3.2)
##	GenomeInfoDbData	1.2.11	2023-12-21 [1] Bioconductor	
##	GenomicAlignments	1.38.2	2024-01-16 [1] Bioconductor 3.18 (F	
##	GenomicFeatures	1.54.3	2024-01-31 [1] Bioconductor 3.18 (F	R 4.3.2)
##	GenomicRanges	1.54.1	2023-10-29 [1] Bioconductor	
##	ggnetwork	0.5.13	2024-02-14 [1] CRAN (R 4.3.2)	
##		3.4.4	2023-10-12 [1] CRAN (R 4.3.2)	
##	glue	1.7.0	2024-01-09 [1] CRAN (R 4.3.2)	
##	gtable	0.3.4	2023-08-21 [1] CRAN (R 4.3.2)	
##	here *	1.0.1	2020-12-13 [1] CRAN (R 4.3.2)	
##	hms	1.1.3	2023-03-21 [1] CRAN (R 4.3.2)	
##	htmltools	0.5.7	2023-11-03 [1] CRAN (R 4.3.2)	
##	htmlwidgets	1.6.4	2023-12-06 [1] CRAN (R 4.3.2)	
##	httr	1.4.7	2023-08-15 [1] CRAN (R 4.3.2)	
##	igraph	2.0.1.1	2024-01-30 [1] CRAN (R 4.3.2)	
##	intergraph	2.0-4	2024-02-01 [1] CRAN (R 4.3.2)	
##	IRanges	2.36.0	2023-10-24 [1] Bioconductor	
##	iterators	1.0.14	2022-02-05 [1] CRAN (R 4.3.2)	
##	KEGGREST	1.42.0	2023-10-24 [1] Bioconductor	
##	knitr	1.45	2023-10-30 [1] CRAN (R 4.3.2)	
##	lattice	0.22-5	2023-10-24 [4] CRAN (R 4.3.1)	
##	lifecycle	1.0.4	2023-11-07 [1] CRAN (R 4.3.2)	
##		1.9.3	2023-09-27 [1] CRAN (R 4.3.2)	
##	magrittr	2.0.3	2022-03-30 [1] CRAN (R 4.3.2)	
##	MASS	7.3-60	2023-05-04 [4] CRAN (R 4.3.1)	
##	Matrix	1.6-3	2023-11-14 [4] CRAN (R 4.3.2)	
##	MatrixGenerics	1.14.0	2023-10-24 [1] Bioconductor	
##	matrixStats	1.2.0	2023-12-11 [1] CRAN (R 4.3.2)	
##	mclust	6.0.1	2023-11-15 [1] CRAN (R 4.3.2)	
##	memoise	2.0.1	2021-11-26 [1] CRAN (R 4.3.2)	
##	MSA2dist	1.6.0	2023-10-24 [1] Bioconductor	
##	munsell	0.5.0	2018-06-12 [1] CRAN (R 4.3.2)	
##	network	1.18.2	2023-12-05 [1] CRAN (R 4.3.2)	
##	networkD3	0.4	2017-03-18 [1] CRAN (R 4.3.2)	
##	nlme	3.1-163	2023-08-09 [4] CRAN (R 4.3.1)	
##	pheatmap	1.0.12	2019-01-04 [1] CRAN (R 4.3.2)	
##	pillar	1.9.0	2023-03-22 [1] CRAN (R 4.3.2)	
##	pkgconfig	2.0.3	2019-09-22 [1] CRAN (R 4.3.2)	
##	png	0.1-8	2022-11-29 [1] CRAN (R 4.3.2)	
##	prettyunits	1.2.0	2023-09-24 [1] CRAN (R 4.3.2)	
##	progress	1.2.3	2023-12-06 [1] CRAN (R 4.3.2)	
##		1.0.2	2023-08-10 [1] CRAN (R 4.3.2)	
##	R6	2.5.1	2021-08-19 [1] CRAN (R 4.3.2) 2021-01-31 [1] CRAN (R 4.3.2)	
##	rappdirs	0.3.3	2021-01-31 [1] CNAN (K 4.3.2)	

```
## RColorBrewer
                                                                                            2022-04-03 [1] CRAN (R 4.3.2)
                                                             1.1-3
                                                           1.0.12
                                                                                          2024-01-09 [1] CRAN (R 4.3.2)
 ## Rcpp
                                                           1.98-1.14 2024-01-09 [1] CRAN (R 4.3.2)
 ## RCurl
                                                  * 2.1.5 2024-01-10 [1] CRAN (R 4.3.2)

0.0.15 2022-06-16 [1] CRAN (R 4.3.2)

0.2.21 2022-01-09 [1] CRAN (R 4.3.2)

1.1.3 2024-01-10 [1] CRAN (R 4.3.2)

2.25 2023-09-18 [1] CRAN (R 4.3.2)

2.0.4 2023-11-05 [1] CRAN (R 4.3.2)
 ## readr
 ## restfulr
 ## rjson
 ## rlang
 ## rmarkdown
## rprojroot 2.0.4 2023-11-05 [1] CRAN (R 4.3.2)

## Rsamtools 2.18.0 2023-10-24 [1] Bioconductor

## RSQLite 2.3.5 2024-01-21 [1] CRAN (R 4.3.2)

## rstudioapi 0.15.0 2023-07-07 [1] CRAN (R 4.3.2)

## rtracklayer 1.62.0 2023-10-24 [1] Bioconductor

## S4Arrays 1.2.0 2023-10-24 [1] Bioconductor

## Sales 0.40.2 2023-11-23 [1] Bioconductor 3.18 (R 4.3.2)

## seqinr 4.2-36 2023-11-28 [1] CRAN (R 4.3.2)

## sessioninfo 1.2.2 2021-12-06 [1] CRAN (R 4.3.2)

## sparseArray 1.2.4 2024-02-11 [1] Bioconductor 3.18 (R 4.3.2)

## stainet.common 4.9.0 2023-05-24 [1] CRAN (R 4.3.2)

## stringi 1.8.3 2023-12-11 [1] CRAN (R 4.3.2)

## stringr * 1.5.1 2023-11-14 [1] CRAN (R 4.3.2)

## stringr * 1.5.1 2023-11-14 [1] CRAN (R 4.3.2)

## summarizedExperiment 1.32.0 2023-10-24 [1] Bioconductor

## syntenet * 1.4.0 2023-10-24 [1] Bioconductor

## tibble * 3.2.1 2023-03-20 [1] CRAN (R 4.3.2)
 ## rprojroot
 ## Rsamtools
 ## synce
## tibble
                                                      * 3.2.1
                                                 * 3.2.1 2023-03-20 [1] CRAN (R 4.3.2)

* 1.3.1 2024-01-24 [1] CRAN (R 4.3.2)

1.2.0 2022-10-10 [1] CRAN (R 4.3.2)

* 2.0.0 2023-02-22 [1] CRAN (R 4.3.2)

0.3.0 2024-01-18 [1] CRAN (R 4.3.2)

0.4.0 2023-05-12 [1] CRAN (R 4.3.2)

1.2.4 2023-10-22 [1] CRAN (R 4.3.2)

0.6.5 2023-12-01 [1] CRAN (R 4.3.2)

3.0.0 2024-01-16 [1] CRAN (R 4.3.2)

0.42 2024-02-08 [1] CRAN (R 4.3.2)

3.99-0.16.1 2024-01-22 [1] CRAN (R 4.3.2)

1.3.6 2023-12-04 [1] CRAN (R 4.3.2)

0.42.0 2023-10-24 [1] Bioconductor

2.3.8 2023-12-11 [1] CRAN (R 4.3.2)

1.48.0 2023-10-24 [1] Bioconductor
                                                                                          2023-03-20 [1] CRAN (R 4.3.2)
 ## tidyselect
 ## tidyverse
 ## timechange
 ## tzdb
 ## utf8
 ## vctrs
 ## withr
 ## xfun
  ## XML
 ## xml2
 ## XVector
 ## yaml
 ## zlibbioc
                                                           1.48.0
                                                                                       2023-10-24 [1] Bioconductor
 ##
  ## [1] /home/faalm/R/x86_64-pc-linux-qnu-library/4.3
  ## [2] /usr/local/lib/R/site-library
 ## [3] /usr/lib/R/site-library
 ## [4] /usr/lib/R/library
  ## -----
```